

FORM PTO-1390 (REV 12-29-99)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 2534US0P
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (if known, see 37 CFR 1.5) <b>09/744226</b>	
INTERNATIONAL APPLICATION NO. PCT/JP99/03909	INTERNATIONAL FILING DATE July 22, 1999		PRIORITY DATE CLAIMED Jul. 23, 1998; Aug. 7, 1998 & Oct. 6, 1998	
TITLE OF INVENTION Novel G Protein Coupled Receptor Protein and Its DNA				
APPLICANT(S) FOR DO/EO/US Osamu OHARA et al.				
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:				
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))           <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ul> </p> <p>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). *</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))           <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input type="checkbox"/> have not been made and will not be made.</li> </ul> </p> <p>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>				
Items 11. to 16. below concern document(s) or information included:				
<p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. (two sets)</p> <p>13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.</p> <p><input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input checked="" type="checkbox"/> Other items or information:           <ul style="list-style-type: none"> <li>1) Sequence Listing (Paper &amp; Computer-readable copies)</li> <li>2) Sequence Listing Statement</li> </ul> </p>				
* This includes specification 136 total pages, including Claims 1-16(2 pgs), Abstract (1 pg), Sequence listing (26 pgs) and Drawings (25 pgs).				
<i>Express Mail EL783844580 VS</i>				

U.S. APPLICATION NO. (if known) 1744226

INTERNATIONAL APPLICATION NO  
PCT/JP99/03909ATTORNEYS DOCKET NUMBER  
2534US0P

17. <input checked="" type="checkbox"/> The following fees are submitted:		<b>CALCULATIONS PTO USE ONLY</b>	
<b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5) ) :</b>			
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$970.00			
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00			
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$690.00			
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$670.00			
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$96.00			
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>		\$ 860.00	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	23 - 20 =	3	X \$18.00
Independent claims	1 - 3 =	0	X \$ 80.0
MULTIPLE DEPENDENT CLAIM(S) (if applicable)		+ \$260.00	\$ 260.00
<b>TOTAL OF ABOVE CALCULATIONS =</b>		\$ 1174.00	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).		\$	
<b>SUBTOTAL =</b>		\$ 1174.00	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).		\$	
<b>TOTAL NATIONAL FEE =</b>		\$ 1174.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property		+ \$ 80.00	
<b>TOTAL FEES ENCLOSED =</b>		\$ 1254.00	
		<b>Amount to be refunded:</b>	\$
		<b>charged:</b>	\$
<p>a. <input type="checkbox"/> A check in the amount of \$ _____ to cover the above fees is enclosed.</p> <p>b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. 500799 in the amount of \$ 1254.00 to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 500799. A duplicate copy of this sheet is enclosed.</p>			
<p><b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b></p>			
SEND ALL CORRESPONDENCE TO: Customer No. 23,115			
 SIGNATURE: Philippe Y. Riesen			
NAME 35,657			
REGISTRATION NUMBER Date: January 12, 2001			

09/744226

J002 Rec'd PCT/PTO 22 JAN 2001  
Attorney Docket No. 2534USOP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Osamu OHARA et al.  
Serial No. : Attn: Box PCT  
Filed on :  
Title : Novel G Protein Coupled Receptor Protein and Its DNA

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Preliminary to examination please amend the above-identified application as follows:

IN THE SPECIFICATION:

Page 1, first sentence, insert "This application is the National Stage of International Application No. PCT/JP99/03909, filed on July 22, 1999."

Page 2, line 1, delete "mean" and substitute therefor --means--; delete "medicals" and substitute therefor --medicines--

line 3, before "regulation" delete "the"

line 13, delete "mean" and substitute therefor --means--; delete "medicals" and substitute therefor --medicines--

line 15, delete "medicals" and substitute therefor --medicines--

line 17, delete "the"

line 21, delete "in" and substitute therefor --of--

Page 3, line 6, delete "property" and substitute therefor --properties--

line 10, delete "property" and substitute therefor --properties--

line 12, delete "studies" and substitute therefor --studied--

line 21, delete "comprises" and substitute therefor --comprising--

line 24, delete "comprises" and substitute therefor --comprising--

Page 4, line 1, delete "comprising" and substitute therefor --comprises--

Page 15, line 20, delete ")))"

Page 16, line 18, delete "An" and substitute therefor --As an--

line 20, delete "are" and substitute therefor --is--

Page 17, line 1, before "a protein" insert --there is--

line 10, delete "an" and substitute therefor --the--

Page 18, line 8, delete "an" and substitute therefor --the--

line 19, delete "in which a" and substitute therefor --and where the--

line 19-20, delete "in the living body" and substitute therefor --in vivo--

Page 20, line 15, before "N-terminal" delete "a" and substitute therefor --the--; delete

"in the living body" and substitute therefor --in vivo--  
Page 21, line 1, delete "hydrocloric" and substitute therefor --hydrochloric--  
line 18, delete "the"  
line 23, delete "the"  
Page 25, line 13, delete "alcohols" and substitute therefor --alcohol--  
Page 27, line 2, delete "the"  
line 15, delete "the"  
Page 28, line 5, delete "the"  
Page 31, line 7, before "group" delete "the" and substitute therefor --a--  
line 14, delete "method" and substitute therefor --methods--  
Page 32, line 4, delete "the"  
Page 33, line 4, delete "These" and substitute therefor --The--  
Page 37, line 9, delete "airation" and substitute therefor --aeration--  
line 19, delete "airation" and substitute therefor --aeration--  
line 24, delete "airation" and substitute therefor --aeration--  
Page 38, line 7, delete "airation" and substitute therefor --aeration--  
Page 39, line 11, delete "acting" and substitute therefor --adding--  
Page 41, line 19, delete "serem" and substitute therefor --serum--  
Page 45, line 13, delete "the" and substitute therefor --a--  
Page 48, line 10, delete "at" and substitute therefor --in--  
line 11, delete "repturing" and substitute therefor --rupturing--  
Page 49, line 3, delete "at" and substitute therefor --in--  
Page 50, line 3, delete "dose" and substitute therefor --does--  
Page 54, line 16, delete "make" and substitute therefor --made--  
line 24, delete "agen" and substitute therefor --agent--  
line 25, delete "alamono" and substitute therefor --akamono--  
Page 55, line 5, delete "sesami" and substitute therefor --sesame--  
line 20, delete "ampul" and substitute therefor --ampule--  
Page 57, line 14, delete "alter" and substitute therefor --alters--  
line 22, delete "the"  
line 25, delete "the"  
Page 61, line 1, delete "at" and substitute therefor --in--  
Page 62, line 2, delete "at" and substitute therefor --in--  
Page 63, line 10, delete "dose" and substitute therefor --does--  
Page 67, line 8, delete "the"  
line 13, before "cell" delete "a"  
line 20, before "similar" delete "the" and substitute therefor --a--  
line 23, before "similar" delete "the" and substitute therefor --a--  
line 25, delete "the"  
Page 70, line 4, delete "in" and substitute therefor --from--  
Page 71, line 16, after "used" insert --in--  
Page 72, line 9, delete "presipitates resulted" and substitute therefor --precipitates

resulting--

line 12, delete "presipitates" and substitute therefor --precipitates--

line 16, delete "to" insert --in--

Page 73, line 8, delete "the"

Page 74, line 4, delete "at" and substitute therefor --in--

line 8, delete "ubiquous" and substitute therefor --ubiquitous--

line 13, delete "an embryo cell and a somatic cell" insert --the embryo cells and somatic cells--

line 16, delete "their all" and substitute therefor --all their--

line 17, after "such" delete "the"

Page 75, line 1, delete "expresses highly" and substitute therefor --highly expresses--

IN THE CLAIMS:

Claim 1, page 83, line 2, delete "comprises" and substitute therefor --comprising--

Claim 3, page 83, line 6, delete "comprises" and substitute therefor --comprising--

Claim 4, page 83, line 8, delete "comprising" and substitute therefor --comprises--

**REMARKS**

The above amendments correct typographical and clerical errors and do not constitute new matter. Entry of the above amendments prior to examination and early action on the merits are respectfully requested.

Date: January 12, 2001

Respectfully submitted,



Philippe Y. Riesen, Reg. No. 35,657  
Attorney for Applicants

Customer No. 23,115

25/PPR3

09/744226

J002 Rec'd PCT/PTO 22 JAN 2001

## SPECIFICATION

### NOVEL G PROTEIN-COUPLED RECEPTOR PROTEIN AND ITS DNA

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#### Technical Field

The present invention relates to a human brain-derived novel protein (G protein-coupled receptor protein) or a salt thereof and a DNA encoding it.

#### 10 Background Technique

Many hormones and neurotransmitters regulate the functions of the living body via specific receptor proteins present in a cell membrane. Many of these receptor proteins perform intracellular signal transmittance through activation of conjugated guanine nucleotide-binding protein (hereinafter abbreviated as G protein in some cases) and, since they have the common structure having seven transmembrane regions, they are collectively called G protein-coupled receptor proteins or seven transmembrane receptor protein.

G protein-coupled receptor protein is present at various functional cell surfaces of cells or organs of the living body and carries an important role as a target for molecules which regulate the functions of cells or organs of the living body, for example, hormones, neurotransmitters and physiologically active substances.

Revelation of the relationship between substances which regulate the complex functions within cells and organs of the various living bodies and specific receptor proteins therefore, in particular, G protein-coupled receptors elucidates the functions of cells and organs of various living bodies and provides a very important

mean for development of medicals related to the functions.

For example, in central nervous system organs, regulation of the physiological functions of the brain is performed under the regulation by many hormones, neurotransmitters or physiologically active substances. In particular, 5 neurotransmitters are present in various sites in the brain and perform the regulation of the physiological functions through each corresponding receptor protein. There are many unknown transmitters in the brain and it is thought that many of the structures of cDNA's encoding receptor proteins have not been reported yet. Further, whether a subtype of the known receptor protein is present or not has not been 10 elucidated.

Revelation of the relationship between substances which regulate the complicated functions within cells and organs of the various living bodies and specific receptor proteins therefore is a very important mean for developing medicals related to the functions. In addition, in order to effectively screen agonists and antagonists for 15 a receptor protein and develop medicals, it is necessary to elucidate the functions of genes for receptor proteins which are expressed in the brain and allow the genes to be expressed in the suitable expressing system.

Recently, as means for analyzing genes expressed in the living body, random analysis of the sequences of cDNA's have been actively studied and the sequences of 20 fragments of cDNA's thus obtained are registered in database as Expressed Sequence Tag (EST) and published. However, many EST's are only information in the sequence and it is difficult to presume the functions.

### Summary of the Invention

25 The present invention provides a human brain-derived novel protein (G

protein-coupled receptor protein), its partial peptide or a salt thereof, a DNA containing  
a DNA encoding the protein or its partial peptide, a recombinant vector containing the  
DNA, a transformant transformed with the recombinant vector, a process for  
producing the protein, an antibody to the protein, its partial peptide or the salt thereof,  
5 a method for determining a ligand for the protein (G protein-coupled receptor protein),  
a method of screening a compound for altering property of a ligand binding with the  
protein (G protein-coupled receptor protein) or a salt thereof, a kit for the screening, a  
compound for altering binding of a ligand obtainable by the screening method or the  
screening kit with the protein (G protein-coupled receptor protein) or a salt thereof, and  
10 a pharmaceutical containing a compound for altering property of a ligand binding with  
the protein (G protein-coupled receptor protein) or a salt thereof.

The present inventors studies intensively and, as a result, we successfully  
isolated a cDNA encoding a human brain-derived novel protein (G protein-coupled  
receptor protein) and analyzed the entire nucleotide sequence. And when this  
15 nucleotide sequence was translated into an amino acid sequence, the first to the  
seventh transmembrane regions were confirmed on a hydrophobic plot and a protein  
encoded by the cDNA was confirmed to be the seventh membrane type G protein-  
coupled receptor protein. The present inventors further continued to study based on  
these findings and, as a result, completed the present invention.

20 That is, the present invention provides:

- (1) A protein comprises the same or substantially the same amino acid sequence  
represented by SEQ ID No:1, SEQ ID No:3 or SEQ ID No:5, or a salt thereof,
- (2) A partial peptide of the protein according to the above item (1), or a salt thereof,
- (3) A DNA comprises a DNA having a nucleotide sequence encoding the protein  
according to the above item (1),

(4) The DNA according to the above item (3), which comprising a nucleotide sequence represented by SEQ ID No:2, SEQ ID No:4 or SEQ ID No:6,

(5) A recombinant vector comprising the DNA according to the above item (3).

(6) A transformant transformed with the recombinant vector according to the above  
5 item (5),

(7) A process for producing the protein or a salt thereof according to the above item (1), which comprises culturing the transformant according to the above item (6), and producing and accumulating the protein according to the above item (1),

10 (8) An antibody to the protein according to the above item (1), the partial peptide according to the above item (2) or a salt thereof,

(9) A method for determining a ligand for the protein according to the above item (1) or a salt thereof, which comprises using the protein according to the above item (1), the partial peptide according to the above item (2) or a salt thereof.

15 (10) A method for screening a compound or a salt thereof which alters property of a ligand binding with the protein according to the above item (1) or a salt thereof, which comprises using the protein to the above item (1), the partial peptide according to the above item (2) or a salt thereof,

20 (11) A kit for screening a compound or a salt thereof which alters property of a ligand binding with the protein according to the above item (1) or a salt thereof, which comprises the protein according to the above item (1), the partial peptide according to the above item (2) or a salt thereof,

25 (12) A compound or a salt thereof which alters property of a ligand binding with the protein according to the above item (1), or a salt thereof, which is obtainable by the method for screening according to the above item (10) or the kit for screening according to the above item (11).

(13) A pharmaceutical which comprises a compound or a salt thereof which alters property of a ligand binding with the protein according to the above item (1) or a salt thereof, which is obtainable by the method for screening according to the above item (10) or the kit for screening according to the above item (11),

5 (14) A DNA which hybridizes with the DNA according to the above item (3) under stringent conditions,

(15) A nucleotide containing a nucleotide sequence encoding the protein according to the above item (1), and

10 (16) A nucleotide containing a part of a nucleotide sequence which is complementary to a nucleotide sequence encoding the protein according to the above item (1).

More particularly, the present invention provides:

(17) The protein or a salt thereof according to the above item (1), wherein the protein is a protein containing:

① an amino acid sequence in which 1 or 2 or more (preferably around 1 - 30, more preferably around 1-9, most preferably a few) amino acids in an amino acid sequence of SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.:5 are deleted, ② an amino acid sequence in which 1 or 2 or more (preferably around 1 - 30, more preferably around 1 - 10, most preferably a few) amino acids are added to an amino acid sequence of SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.:5, ③ an amino acid sequence in which 1 or 2 or more (preferably around 1 - 30, more preferably around 1-10, most preferably a few) amino acids in an amino acid sequence of SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.:5 are substituted with other amino acids, or ④ an amino acid sequence in combination thereof,

(18) A method for determining the ligand according to the above item (9), which 25 comprises contacting the protein or a salt thereof according to the above item (1) or

the partial peptide or a salt thereof according to the above item (2) with a test compound,

(19) A method for determining the ligand according to the above item (9), wherein the ligand is angiotensin, bombesin, cannabinoid, cholecystokinin, glutamine, serotonin,

5 melatonin, neuropeptide Y, opioid, purine, vasopressin, oxytocin, PACAP, secretin, glucagon, calcitonin, adrenomedulin, somatostatin, GHRH, CRF, ACTH, GRP, PTH, VIP (vasoactive intestinal and related polypeptide), somatostatin, dopamine, motilin, amylin, bradykinin, CGRP (calcitonin gene related peptide), leukotriene, pancreastatin, prostaglandin, thromboxane, adenosine, adrenaline,  $\alpha$  and  $\beta$ -chemokine (for example, IL-8, GRO $\alpha$ , GRO $\beta$ , GRO $\gamma$ , NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, 10 MCP-3, I-309, MIP1 $\alpha$ , MIP-1 $\beta$ , RANTES and the like), endothelin, enterogastrin, histamine, neurotensin, TRH, pancreatic polypeptide, galanin,  $\alpha$ -latrotoxin, neurexophilin, subtype thereof, or analogue thereof,

(20) The method for determining a ligand according to the above item (9), wherein the

15 ligand is  $\alpha$ -latrotoxin, neurexophilin, subtype thereof, or analogue thereof,

(21) The screening method according to the above item (10), wherein (i) the case

where the protein or a salt thereof according to the above item (1) or the partial

peptide or a salt thereof according to the above item (2) is contacted with a ligand, and

(ii) the case where the protein or a salt thereof according to the above item (1) or the

20 partial peptide or a salt thereof according to the above item (2) is contacted with a ligand and a test compound are compared,

(22) A method for screening a compound or a salt thereof which alters property of a

ligand binding with the protein or a salt thereof according to the above item (1), which

comprises measuring and comparing an amount of a labeled ligand binding to the

25 protein or a salt thereof according to the above item (1) or the partial peptide or a salt

thereof according to the above item (2) in (i) the case where a labeled ligand is contacted with the protein or a salt thereof according to the above item (1) or the partial peptide or a salt thereof according to the above item (2) and the amount in (ii) the case where a labeled ligand and a test compound are contacted with the protein or 5 a salt thereof according to the above item (1) or the partial peptide or a salt thereof according to the above item (3),

(23) A method for screening a compound or a salt thereof which alters property of a ligand binding with the protein or a salt thereof according to the above item (1), which comprises measuring and comparing an amount of a labeled ligand binding with a cell 10 having the protein according to the above item (1) in (i) the case where a labeled ligand is contacted with the cell, and the amount in (ii) the case where a labeled ligand and a test compound are contacted with the cell,

(24) A method for screening a compound or a salt thereof which alters property of a ligand binding with the protein or a salt thereof according to the above item (1) which comprises measuring and comparing an amount of a labeled ligand binding with a 15 membrane fraction of a cell having the protein according to the above item (1) in (i) the case where a labeled ligand is contacted with the cell, and the amount in (ii) the case where a labeled ligand and a test compound are contacted with the fraction,

(25) A method for screening a compound or a salt thereof which alters property of a ligand binding with the protein or a salt thereof according to the above item (1) which comprises measuring and comparing an amount of a labeled ligand binding with a 20 protein expressed on a cell membrane of the transformant according to the above item (6) in (i) the case where a labeled ligand is contacted with the protein by culturing the transformant, and the amount in (ii) the case where a labeled ligand and a test 25 compound are contacted with the protein by culturing the transformant,

(26) A method for screening a compound or a salt thereof which alters property of a ligand binding with the protein or a salt thereof according to the above item (1), which comprises measuring and comparing the cell stimulating activity via a protein in (i) the case where a compound which activates the protein or a salt thereof according to  
5 the above item (1) is contacted with a cell containing the protein according to the above item (1), and the activity in (ii) the case where the protein or a salt thereof according to the above item (1) and a test compound are contacted with a cell containing the protein according to the above item (1),

(27) A method for screening a compound or a salt thereof which alters property of a ligand binding with the protein or a salt thereof according to the above item (1), which comprises measuring and comparing the cell stimulating activity via a protein expressed in the transformant according to the above item (6) in (i) the case where a compound or a salt thereof which activates the protein or a salt thereof according to the above item (1) is contacted with the protein by culturing the transformant  
10 according to the above item (6), and the activity in (ii) the case where a compound which activates the protein or a salt thereof according to the above item (1) and a test compound are contacted with the protein by culturing the transformant according to the above item (6),  
15

(28) The screening method according to the above item (26) or (27), wherein the compound which activates the protein according to the above item (1) is angiotensin,  
20 bombesin, cannabinoid, cholecystokinin, glutamine, serotonin, melatonin, neuropeptide Y, opioid, purine, vasopressin, oxytocin, PACAP, secretin, glucagon, calcitonin, adrenomedulin, somatostatin, GHRH, CRF, ACTH, GRP, PTH, VIP (vasoactive intestinal and related polypeptide), somatostatin, dopamine, motilin, amylin,  
25 bradykinin, CGRP (calcitonin gene related peptide), leukotriene, pancreastatin,

prostaglandin, thromboxane, adenosine, adrenaline,  $\alpha$  and  $\beta$ -chemokine (for example, IL-8, GRO $\alpha$ , GRO $\beta$ , GRO $\gamma$ , NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, MCP-3, I-309, MIP1 $\alpha$ , MIP-1 $\beta$ , RANTES and the like), endothelin, enterogastrin, histamine, neuropeptides, TRH, pancreatic polypeptide, galanin,  $\alpha$ -latrotoxin,

5 neurexophilin, subtype thereof, or analogue thereof,

(29) The screening method according to the above item (26) or (27), wherein the compound which activates the protein according to the above item (1) is  $\alpha$ -latrotoxin, neurexophilin, a subtype thereof, or an analogue thereof,

10 (30) A compound which alters property of a ligand binding with the protein or a salt thereof, which is obtainable by the screening method according to the above item (21) to (28), or a salt thereof,

15 (31) A pharmaceutical which comprises a compound which alters property of a ligand binding with the protein or a salt thereof according to the above item (1), which is obtainable by the screening method according to the above item (21) to (28), or a salt thereof,

(32) A kit for the screening according to the above item (11), which comprises a cell containing the protein according to the above item (1),

(33) A kit for the screening according to the above item (11), which comprises a membrane fraction of a cell containing the protein according to the above item (1),

20 (34) A kit for the screening according to the above item (11), which comprises a protein expressed on a cell membrane of the transformant according to the above item (6) by culturing the transformant,

(35) A compound which alters property of a ligand binding with the protein or a salt thereof, which is obtainable by the kit for screening according to the above item (32) to (34), or a salt thereof,

(36) A pharmaceutical which comprises a compound which alters property of a ligand binding with the protein or a salt thereof according to the above item (1), which is obtainable by the kit for screening according to the above item (32) to (34), or a salt thereof,

5 (37) A method for quantitating the protein according to the above item (1), the partial peptide according to the above item (2) or a salt thereof, which comprises contacting the antibody according to the above item (8) with the protein according to the above item (1), the partial peptide according to the above item (2) or a salt thereof,

10 (38) A method for quantitating the protein according to the above item (1), the partial peptide according to the above item (2) or a salt thereof in a specimen solution, which comprises competitively reacting the antibody according to the above item (8) with the test solution and the labeled protein according to the above item (1), the partial peptide according to the above item (2) or a salt thereof, and determining a ratio of the protein according to the above item (1), the partial peptide according to the above item (2) or a salt thereof which is bound to the antibody,

15 (39) A method for quantitating the protein according to the above item (1), the partial peptide according to the above item (2) or a salt thereof in a specimen solution, which comprises reacting the specimen solution with the antibody according to the above item (8) and the labeled antibody according to the above item (8) which is unsolubilized on a carrier simultaneously or successively, and determining the activity 20 of a labeling agent on the unsolubilized carrier.

#### Brief Description of the Drawings

Fig. 1 shows a nucleotide sequence encoding a human brain-derived protein

25 of the present invention obtained in Example 1 and an amino acid sequence predicted

therefrom (continued to Fig. 2).

Fig. 2 shows a nucleotide sequence encoding a human brain-derived protein of the present invention obtained in Example 1 and an amino acid sequence predicted therefrom (continued from Fig. 1).

5 Fig. 3 shows a hydrophobic plot of a human brain-derived protein of the present invention, which was made based on the amino acid sequences shown in Fig. 1 and Fig. 2. Parts designated by 1 - 7 show a hydrophobic domain.

10 Fig. 4 shows an amino acid sequence predicted from a nucleotide sequence encoding a human brain-derived protein of the present invention obtained in Example 1 (a sequence represented by HK05006 in the figure) and an amino acid sequence predicted from a nucleotide sequence encoding a human brain-derived protein of the present invention obtained in Example 2 (a sequence represented by HK05490 in the figure)(continued to Fig. 5).

15 Fig. 5 shows an amino acid sequence predicted from a nucleotide sequence encoding a human brain-derived protein of the present invention obtained in Example 1 (a sequence represented by HK05006 in the figure) and an amino acid sequence predicted from a nucleotide sequence encoding a human brain-derived protein of the present invention obtained in Example 2 (a sequence represented by HK05490 in the figure)(continued from Fig. 4).

20 Fig. 6 shows a hydrophobic plot of a human brain-derived protein of the present invention, which was made based on the amino acid sequence predicted based on a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2. Part designated by 1 - 7 show a hydrophobic domain.

25 Fig. 7 shows a nucleotide sequence of a DNA encoding a human brain-

derived protein of the present invention obtained in Example 2, and an amino acid sequence predicted therefrom (continued to Fig. 8).

Fig. 8 shows a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2, and an amino acid sequence predicted therefrom (continued from Fig. 7 and continued to Fig. 9).  
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Fig. 9 shows a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2, and an amino acid sequence predicted therefrom (continued from Fig. 8 and continued to Fig. 10).

Fig. 10 shows a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2, and an amino acid sequence predicted therefrom (continued from Fig. 9 and continued to Fig. 11).  
10

Fig. 11 shows a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2, and an amino acid sequence predicted therefrom (continued from Fig. 10 and continued to Fig. 12).  
15

Fig. 12 shows a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2, and an amino acid sequence predicted therefrom (continued from Fig. 11 and continued to Fig. 13).

Fig. 13 shows a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2, and an amino acid sequence predicted therefrom (continued from Fig. 12 and continued to Fig. 14).  
20

Fig. 14 shows a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2, and an amino acid sequence predicted therefrom (continued from Fig. 13 and continued to Fig. 15).

Fig. 15 shows a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2, and an amino acid  
25

sequence predicted therefrom (continued from Fig. 14).

Fig. 16 shows an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 1 (a sequence represented by HK05006 in the figure), an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2 (a sequence represented by HK05490 in the figure) and an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 3 (a sequence represented by HH02631 in the figure)(continued to Fig.17).

Fig. 17 shows an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 1 (a sequence represented by HK05006 in the figure), an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2 (a sequence represented by HK05490 in the figure) and an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 3 (a sequence represented by HH02631 in the figure)(continued to Fig.18).

Fig. 18 shows an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 1 (a sequence represented by HK05006 in the figure), an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2 (a sequence represented by HK05490 in the figure) and an amino acid sequence predicted from a

nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 3 (a sequence represented by HH02631 in the figure)(continued to Fig.19).

Fig. 19 shows an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 1 (a sequence represented by HK05006 in the figure), an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2 (a sequence represented by HK05490 in the figure) and an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 3 (a sequence represented by HH02631 in the figure)(continued to Fig.20).

Fig. 20 shows an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 1 (a sequence represented by HK05006 in the figure), an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 2 (a sequence represented by HK05490 in the figure) and an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 3 (a sequence represented by HH02631 in the figure)(continued from Fig.19).

Fig. 21 shows a nucleotide sequence contained in the plasmid pHH02631 obtained in Example 3 (continued to Fig. 22).

Fig. 22 shows a nucleotide sequence contained in the plasmid pHH02631 obtained in Example 3 (continued to Fig. 23).

Fig. 23 shows a nucleotide sequence contained in the plasmid pHH02631 obtained in Example 3 (continued to Fig. 24).

Fig. 24 shows a nucleotide sequence contained in the plasmid pHH02631 obtained in Example 3 (continued from Fig. 23).

5       Fig. 25 shows a hydrophobic plot of a human brain-derived protein of the present invention, which was made based on an amino acid sequence predicted from a nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention obtained in Example 3. Parts designated by 1 - 7 show a hydrophobic domain.

10

#### Best Mode for Carrying Out the Invention

A protein of the present invention (G protein-coupled receptor protein) is a receptor protein containing the same or substantially the same amino acid sequence as amino acid sequence represented by SEQ ID No.:1 [amino acid sequence of Fig. 1 and Fig. 2], an amino acid sequence represented by SEQ ID No.:3 [amino acid sequence represented by HK05490 in Fig.4 and Fig. 5; an amino acid sequence in Fig.7 to Fig. 15] or an amino acid sequence represented by SEQ ID No.:5 [amino acid sequence represented by HH02631 Fig.16 to Fig.20] (hereinafter a protein of the present invention (G protein-coupled receptor protein) or a salt thereof is abbreviated as 20 present protein in some cases))).

Present protein (G protein conjugated type protein) may be derived from, for example, in cases of human being and mammal (for example, guinea pig, rat, mouse, rabbit, pig, sheep, cow, monkey and the like), any cell (for example, spleen cell, nerve cell, glia cell, pancreatic  $\beta$  cell, marrow cell, mesangial cell, Langerhans's cell, 25 epidermic cell, epithelial cell, endothelial cell, fibroblast, fibrocyte, myocyte, fat cell,

immunocyte (for example, macrophage, T cell, B cell, natural killer cell, mast cell, neutrophil, basophil, eosinophil, monocyte), megakaryocyte, synovial cell, chondrocyte, bone cell, osteoblast, osteoclast, mammary cell, hepatic cell or interstitial cell, or precursor cell, stem cell or cancer cell thereof) and blood cell (for 5 example, MEL, M1, CTLL-2, HT-2, WEHI-3, HL-60, JOSK-1, K562, ML-1, MOLT-3, MOLT-4, MOLT-10, CCRF-CEM, TALL-1, Jurkat, CCRT-HSB-2, KE-37, SKW-3, HUT-78, HUT-102, H9, U937, THP-1, HEL, JK-1, CMK, KO-812, MEG-01 and the like), or any tissue in which the above cells are present, for example, brain, each site of brain (bulb olfactorius, tonsil nucleus, cerebral basal bulb, hippocampus, 10 thalamus, hypothalamus, subthalamic nucleus, cerebral cortex, meddula oblongata, cerebellum, occipital lobe, frontal lobe, temporal lobe, putamen, caudatum, corpus callosum, nigra), spinal cord, pituitary gland, stomach, pancreas, kidney, liver, gonad, thyroid gland, cholecystis, bone marrow, drenal gland, skin, muscle, lung, alimentary tract (for example, large intestine, small intestine), blood vessel, cardiac thymus, 15 spleen, submadibular gland, peripheral blood, peripheral blood cell, prostate, testis, testicle, ovary, placenta, uterus, bone, joint, skeletal muscle (in particular, brain and respective sites of brain), or a synthetic protein.

An amino acid sequence which is the same or substantially the same as an amino acid sequence represented by SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.: 5, 20 there are an amino acid sequence having about 50% or more, preferably about 70% or more, more preferably about 80% or more, further preferably about 90% or more, most preferably about 95% or more homology with an amino acid sequence represented by SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.: 5.

As a protein having an amino acid sequence which is the same or 25 substantially the same as an amino acid sequence represented by SEQ ID No.:1, SEQ

ID No.:3 or SEQ ID No.: 5, a protein having the substantially same amino acid sequence as an amino acid sequence represented by SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.: 5 and having substantially the same nature of activity as that of an amino acid sequence represented by SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.: 5.

5 As the activity of substantially the same nature, for example, there is a ligand binding property, signal information transmitting action and the like. "Substantially the same" denotes that their activities are the same in nature. Therefore, it is preferable that the activities such as ligand binding activity and signal information transmitting action are equivalent (for example, about 0.5-2-fold) but quantitative 10 elements such as an extent of these activities and a molecular weight of a protein may 15 be different.

Measurement of the activities such as ligand binding and signal transmitting action can be performed according to the method known per se but, for example, measurement can be performed according to a method for determining a ligand or a 15 method for screening described below.

In addition, as present protein, proteins having ① an amino acid sequence in which 1 or 2 or more (preferably around 1 - 30, more preferably around 1 - 10, further preferably a few (1 or 2)) amino acids in an amino acid sequence represented by SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.: 5 are deleted, ② an amino acid sequence in 20 which 1 or 2 or more (preferably around 1 - 30, more preferably around 1 - 10, further preferably a few (1 or 2)) amino acids are added to an amino acid sequence represented by SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.: 5, ③ an amino acid sequence in which 1 or 2 or more (preferably around 1 - 30, more preferably around 1 - 10, further preferably a few (1 or 2)) amino acids in an amino acid sequence 25 represented by SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.: 5 are substituted with an

another amino acid, or ④ amino acid sequence in combination thereof may be used.

In present protein, a left end is a N-terminal (amino terminal) and a right end is a C-terminal (carboxyl terminal) according to the usual convention. In present protein including a protein having an amino acid sequence represented by SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.:5, a C-terminal is usually a carboxyl group (-COOH) or carboxylate (-COO<sup>-</sup>) but a C-terminal may be amide (-CONH<sub>2</sub>) or ester (-COOR).

As R in an ester, C<sub>1-6</sub> alkyl group such as methyl, ethyl, n-propyl, isopropyl and n-butyl, C<sub>3-8</sub> cycloalkyl group such as cyclopentyl and cyclohexyl, C<sub>6-12</sub> aryl group such as phenyl and α-naphthyl, phenyl-C<sub>1-2</sub> alkyl group such as benzyl and phenethyl, or C<sub>7-14</sub> aralkyl group such as α-naphthyl-C<sub>1-2</sub> alkyl group such as α-naphthylmethyl as well as pivaloyloxymethyl group which is used for an oral ester may be used.

When present protein has a carboxyl group (carboxylate) at a site other than a terminal, a protein in which a carboxyl group is amidated or esterified is contained in present protein. As an ester of this case, for example, the aforementioned C-terminal ester and the like are used.

Further, the aforementioned protein in which an amino group of a methionine residue at a N-terminal is protected with a protecting group (for example, C<sub>1-6</sub> acyl group such as formyl group, acetyl group), in which a N-terminal side is cut in the living body and the produced glutamyl group is pyroglutamine-oxidized, or in which a substituent (for example, -OH, -COOH, amino group, imidazole group, indole group, guanidino group) on a side chain of an intramolecular amino acid is protected with a suitable protecting group (for example, C<sub>1-6</sub> acyl group such as formyl group, acetyl group), and a glucoprotein in which a sugar chain is bound are included in present protein.

As an embodiment of present protein, for example, a human-derived (more preferably human brain-derived) protein having an amino acid sequence represented by SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.:5 and the like are used.

As a partial peptide of present protein (hereinafter referred to as partial peptide in some cases), any partial peptide may be used as long as it is a partial peptide of the aforementioned present protein, for example, a site which is protruding outside a cell membrane and has a receptor activity within present protein is used.

More particularly, as a partial peptide of a protein having an amino acid sequence represented by SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.:5, it is a peptide containing a part which is analyzed to be an extracellular region (hydrophilic site) in hydrophobic plot analysis shown by [Fig.3], [Fig.6] or [Fig. 25]. In addition, a peptide containing a hydrophobic region in its part may be also used. A peptide containing separately an individual domain may be used, a peptide of a part containing a plurality of domains at the same time may be used.

Regarding the number of amino acids of a partial peptide of the present invention, a peptide having at least 20 or more, preferably 50 or more, more preferably 100 or more amino acid sequences among component amino acid sequences of the aforementioned present protein is preferable.

“Substantially the same amino acid sequence” denotes an amino acid sequence having about 50% or more, preferably about 70% or more, more preferably about 80% or more, further preferably about 90% or more, most preferably about 95% or more homology with these amino acid sequences.

Here, “substantially the same nature of activity” is as defined above. Measurement of “substantially the same nature of activity” can be performed as described above.

In addition, in a partial peptide of the present invention, 1 or 2 or more (preferably around 1 - 10, more preferably a few (1 or 2) amino acids may be deleted from the aforementioned amino acid sequence, 1 or 2 or more (preferably around 1 - 20, more preferably around 1 - 10, further preferably a few (1 or 2) amino acids may 5 be added to the aforementioned amino acid sequence, or 1 or 2 or more (preferably around 1 - 10, more preferably around 1 - 5, further preferably a few (1 or 2) amino acids in the aforementioned amino acid sequence may be substituted with an another amino acid.

In addition, in a partial peptide of the present invention, a C-terminal is 10 usually a carboxyl group (-COOH) or carboxylate (-COO<sup>-</sup>) but, as in the aforementioned present protein, a C-terminal may be amide (-CONH<sub>2</sub>) or ester (-COOR).

Further, as in the aforementioned present protein, a partial peptide in which an amino group of a methionine residue of a N-terminal is protected with a protecting 15 group, a partial peptide in which a N-terminal side is cut in the living body and the produced Gln is pyroglutamine-oxidized, a partial peptide in which a substituent on a side chain of an intramolecular amino acid is protected with a suitable protecting group, and a composite peptide such as a glucopeptide in which a sugar chain is bound thereto are included in a partial peptide of the present invention.

20 In addition, in a partial peptide of the present invention, a C-terminal is usually a carboxyl group (-COOH) or carboxylate (-COO<sup>-</sup>) but, as in the aforementioned present protein, a C-terminal may be amide (-CONH<sub>2</sub>) or ester (-COOR).

As salts of present protein or a partial peptide thereof, inter alia, 25 physiologically acceptable acid addition salts are preferable. As such the salt, for

example, salts with inorganic acids (such as hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid), salts with organic acids (such as acetic acid, formic acid, propioic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) and  
5 the like are used.

Present protein or a salt thereof can be prepared from the aforementioned cells or tissues derived from human or a mammal by the method of purifying a protein which is known per se, or may be prepared by culturing a transformant containing a DNA encoding present protein described below. Alternatively, the protein may be  
10 prepared by a method for synthesizing a protein described below or a similar method.

When prepared from tissues or cells of human being or a mammal, tissues or cells of human being or a mammal are homogenized and, thereafter, extraction is performed with an acid or the like, and the extract may be purified and isolated by combining chromatography such as reverse phase chromatography, ion-exchange  
15 chromatography and the like.

In order to synthesize present protein, or a partial peptide thereof or a salt thereof or an amide thereof, a commercially available resin for synthesizing a protein may be usually used. As such the resin, for example, there are chloromethyl resin, hydroxymethyl resin, benzhydrylamine resin, aminomethyl resin, 4-  
20 benzyloxybenzylalcohol resin, 4-methylbenzhydrylamine resin, PAM resin, 4-hydroxymethylmethylphenylacetamidemethyl resin, polyacrylamide resin, 4-(2',4'-dimethoxyphenyl-hydroxymethyl)phenoxy resin, 4-(2',4'-dimethoxyphenyl-Fmoc aminoethyl)phenoxy resin and the like. By using such the resin,  $\alpha$ -amino acid and an amino acid having a side functional group suitably protected are condensed on a resin  
25 as a sequence of a protein of interest according to various condensing methods which

are known per se. At the end of a reaction, a protein is cut from a resin and at the same time various protecting groups are removed and, further, an intramolecular disulfide linkage forming reaction is performed and a protein of interest or an amide thereof is obtained.

5 As the aforementioned condensation of a protected amino acid, although various activating reagents which can be used for synthesizing a protein may be used, in particular, carbodiimides are better. As carbodiimides, DCC, N',N'-diisopropylcarbodiimide, N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide and the like are used. For activation by them, a protected amino acid is directly added to a 10 resin together with a racemization inhibiting agent (for example, HOEt, HOOEt), or after a protected amino acid is activated in advance as a symmetrical anhydride or HOEt ester or HOOEt ester, it may be added to a resin.

A solvent used for activating a protected amino acid or condensing with a resin may be appropriately selected from solvents which are known to be used for a 15 protein synthesizing reaction. For example, acid amides such as N,N-dimethylformamide, N,N-dimethylacetamide, N-methylpyrrolidone and the like, halogenated hydrocarbons such as methyl chloride, chloroform and the like, alcohols such as trifluoroethanol and the like, sulfoxides such as dimethyl sulfoxide and the like, ethers such as pyridine, dioxane, tetrahydrofuran and the like, nitriles such as 20 acetonitrile, propionitrile and the like, esters such as methyl acetate, ethyl acetate and the like or an appropriate mixture thereof may be used. A reaction temperature is appropriately selected from a range which is known to be used for a protein binding forming reaction and is usually selected from a range of about -20°C to 50°C. An activated amino acid derivative is usually used at 1.5-4 fold excess amount. When 25 condensation is insufficient as a result of a test using a ninhydrin reaction, sufficient

condensation can be performed by repeating a condensation reaction without leaving a protecting group. When sufficient condensation is not obtained even by repeating a reaction, an unreacted amino acid may be acetylated using acetic anhydride or acetylimidazole.

5 As a protecting group for a raw material amino acid, for example, Z, Boc, tertiary pentylcarbonyl, isobornyloxycarbonyl, 4-methoxybenzyloxycarbonyl, Cl-Z, Br-Z, adamantlyloxycarbonyl, trifluoroacetyl, phthaloyl, formyl, 2-nitrophenylsulfonyl, diphenylphosphinothioyl, Fmoc and the like are used.

A carboxyl group can be protected by, for example, alkylesterification  
10 (straight, branched or cyclic alkylesterification such as methyl, ethyl, propyl, butyl, tertiary-butyl, cyclopentyl, cyclohexyl, cyclopentyl, cyclooctyl, 2-adamantyl and the like), aralkylesterification (for example, benzylester, 4-nitrobenzylester, 4-methoxybenzylester, 4-chlorobenzylester, benzhydrylesterification), phenacylesterification, benzyloxycarbonylhydrizidation, tertiary-  
15 butoxycarbonylhydrizidation, tritylhydrizidation and the like.

A hydroxy group of serine can be protected by, for example, esterification or etherification. As a group suitable for this esterification, for example, lower alkanoyl group such as acetyl group, aroyl group such as benzoyl group, a group derived from carbonic acid such as benzyloxycarbonyl group, ethoxycarbonyl group  
20 and the like are used. In addition, a group suitable for etherification, for example, there are benzyl group, tetrahydropyranyl group, t-butyl group and the like.

As a protecting group for a phenolic hydroxy group of thyrosine, for example, Bzl, Cl<sub>2</sub>-Bzl, 2-nitobenzyl, Br-Z, tertiary-butyl and the like are used.

As a protecting group for imidazole of histidine, for example, Tos, 4-methoxy-2,3,6-trimethylbenzenesulfonyl, DNP, benzyloxymethyl, Bum, Boc, Trt,  
25

Fmoc and the like are used.

As activated carboxyl group of a raw material, for example, corresponding anhydride, azide, active ester [ester with alcohol (for example, pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrophenol, cyanomethylalcohol, para-nitrophenol, 5 HONB, N-hydroxysuccinimide, N-hydroxyphthalimide, HOBT)] and the like are used.

As an activated amino group of a raw material, for example, corresponding phosphoric amide is used.

As a method of removing (eliminating) a protecting group, for example, catalytic reduction in a hydrogen stream in the presence of a catalyst such as Pd-black or Pd-carbon, treatment with an acid such as anhydrous hydrofluoric acid, methanesulfonic acid, trifluoromethanesulfonic acid, trifluoroacetic acid or a mixture thereof, treatment with a base such as diisopropylethylamine, triethylamine, piperidine and the like, and reduction with sodium in liquid ammonia are used. In the acid treatment, for example, the addition of a cation capturing agent such as anisole, phenol, thioanisole, methacresol, paracresol, dimethyl sulfide, 1,4-butanediol, 1,2-ethanedithiol and the like is effective. In addition, 2,4-dinitrophenyl group used as a protecting group for imidazole of histidine is removed by treatment with thiophenol, and formyl group used as a protecting group for indole of tryptophan is also removed by alkali treatment with an aqueous dilute solution of sodium hydroxide, dilute ammonia and the like, in addition to deprotection by the aforementioned acid treatment in the presence of 1,2-ethanedithiol, 1,4-butanedithiol and the like.

Protection of functional groups which should not be involved in a reaction of a raw material and protecting group, elimination of the protecting groups, and activation of functional groups which should be involved in a reaction can be 25 appropriately selected from the known groups and the known means.

DRAFTING PRACTICE

As an another method for obtaining an amide of a protein, for example, an  $\alpha$ -carboxyl group of a carboxyl terminal amino acid is first amidated for protection and, thereafter, a peptide (protein) chain is extended to a desired chain length on an amino group side, a protein in which only a protecting group for a N-terminal  $\alpha$ -amino acid 5 of the peptide chain is removed and a protein in which only a protecting group for a C-terminal carboxyl group is removed are prepared, and both proteins are condensed in the aforementioned mixed solvent. The details of a condensing reaction are as defined above. After a protected protein obtained by condensation is purified, all the 10 protecting groups can be removed by the aforementioned method to obtain a crude desired protein. The crude protein can be purified by the known various purifying means and main fractions can be lyophilized to obtain an amide of a desired protein.

In order to obtain an ester, for example, after a  $\alpha$ -carboxyl group of a carboxy terminal amino acid is condensed with a desired alcohols to make an amino acid ester, an ester of a desired protein can be obtained as in an amide of a protein.

15 Present protein and a salt thereof can be prepared according to the method of synthesizing a peptide which is known per se, or by cutting present protein with a suitable peptidase. A method of synthesizing a peptide may be by a solid phase synthesizing method or a solution phase synthesizing method. That is, a partial peptide or amino acids which can constitute present protein can be condensed with the 20 remaining part and, when a product has a protecting group, a protecting group can be eliminated to prepare a peptide of interest. As the known condensing method and elimination of a protecting group, for example, there are methods described in the following ①-⑤.

① M. Bondanszky and M.A. Ondetti, Peptide Synthesis, Interscience

25 Publishers, New York (1966),

- ② Schroeder and Luebke, *The Peptide*, Academic Press, New York (1965),  
③ Nobuo Izumiya and others, *Fundament and Experiment of Peptide  
Synthesis*, Maruzen (K.K.)(1975)
- ④ Haruaki Yajima and Shunpei Sakakibara, *Biochemistry Experiment 1,  
Chemistry for Proteins IV*, 205, (1977),  
⑤ Supervised by Haruaki Yajima, *Development of Drugs, Second Series,  
vol.14, Peptide Synthesis*, Hirokawa Shoten.

In addition, after a reaction, a partial peptide of the present invention can be purified and isolated by combining, for example, solvent extraction, distillation, column chromatography, liquid chromatography, recrystallization and the like.

When a partial peptide obtained by the aforementioned method is a free compound, it can be converted into a suitable salt by the known method and, conversely, when it is obtained as a salt, it can be converted into a free compound by the known method.

As a DNA encoding present protein, it may be any DNA as long as it contains a nucleotide sequence encoding the aforementioned present protein. In addition, it may be any of a genomic DNA, a genomic DNA library, a cDNA derived from the aforementioned cells or tissues, a cDNA library derived from the aforementioned cells or tissues, and a synthetic DNA. A vector used in a library may be any of bacteriophage, plasmid, cosmid, phagemide and the like. In addition, a total RNA or a mRNA fraction is prepared from the aforementioned cells or tissues, which may be used to directly amplify by Reverse Transcriptase Polymerase Chain Reaction (hereinafter, abbreviated as RT-PCR method).

More particularly, a DNA encoding present protein may be, for example, a DNA having a nucleotide sequence which hybridizes with a nucleotide sequence represented by SEQ ID No.:2, SEQ ID No.:4 or SEQ ID No.:6, or any DNA having a

nucleotide sequence which hybridizes with a nucleotide sequence represented by SEQ ID No.:2, SEQ ID No.:4 or SEQ ID No.:6 under the stringent conditions and encoding a protein having substantially the same nature of activity (for example, ligand binding activity, signal information transmitting action and the like) as that of present protein.

5 As a DNA which can hybridize with a nucleotide sequence represented by SEQ ID No.:2, SEQ ID No.:4 or SEQ ID No.:6, a DNA having a nucleotide sequence having about 70% or more, preferably about 80% or more, more preferably about 90% or more, most preferably about 95% or more homology with a nucleotide sequence represented by SEQ ID No.:2, SEQ ID No.:4 or SEQ ID No.:6 is used.

10 Hybridization can be performed according to the method known per se or a similar method, for example, a method described in Molecular Cloning, 2nd (J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989). In addition, when a commercially available library is used, hybridization can be performed by a method described in the attached specification. More preferably, hybridization can be  
15 performed according to the high stringent conditions.

High stringent conditions show the conditions in which the sodium concentration is about 19-40 mM, preferably about 19-20 mM, and a temperature is about 50-70°C, preferably about 60-65°C. In particular, the case where the sodium concentration is about 19 mM and a temperature is about 65°C is the most preferable.

20 More particularly, as a DNA encoding a protein having an amino acid sequence represented by SEQ ID No.:1, SEQ ID No.:3 or SEQ ID No.:5, a DNA having a nucleotide sequence represented by SEQ ID No.:2, SEQ ID No.:4 or SEQ ID No.:6 is used.

A nucleotide (oligonucleotide) a nucleotide sequence having a nucleotide sequence encoding present protein, or having a part of a nucleotide sequence

complementary to the aforementioned nucleotide sequence is meant to include not only a DNA but also a RNA encoding present protein or a partial peptide thereof.

According to the present invention, an antisense • (oligo)nuclotide (nucleic acid) can be designed and synthesized based on a nucleotide sequence information of 5 a nucleotide sequence encoding a cloned or sequenced protein. Such the (oligo)nuclotide (nucleic acid) can hybridize with a RNA of a G protein coupled receptor protein gene. Synthesis or function of the RNA can be inhibited, or expression of a G protein coupled receptor protein gene can be regulated and controlled via interaction with a G protein coupled receptor protein related RNA. An 10 (oligo)nuclotide complementary to a selected sequence of a G protein coupled receptor protein related RNA, and an (oligo)nuclotide which can specifically hybridize with a G protein coupled receptor protein related RNA are useful for regulating and controlling expression of a G protein coupled receptor protein gene in or outside the living body and also useful for treating or diagnosing diseases.

15 Term “corresponding” means to have homology with or complementary to a particular sequence of a nucleotide, a nucleotide sequence or a nucleic acid including a gene. “Corresponding” between a nucleotide, a nucleotide sequence or a nucleic acid and a peptide (protein) denotes an amino acid of a peptide (protein) under direction derived from a nucleotide (nucleic acid) sequence or a complementary 20 sequence thereof. Although 5' terminal hairpin loop, 5' terminal 6 base pair repeat, 5' terminal untranslated region, polypeptide translation initiation codon, protein coding region, ORF translation initiation codon, 3'terminal untranslated region, 3'terminal palindrome region, and 3'terminal hairpin loop can be selected as a preferable target region, any region within a G protein conjugated type protein gene can be selected as a 25 target.

Relationship between a nucleic acid of interest and an (oligo)nucleotide

which is complementary to at least a part of a target region, and relationship between a subject and an (oligo)nucleotide which can hybridize with the subject can be said to be "antisense". As an antisense (oligo)nucleotide, there are a polydeoxynucleotide  
5 containing 2-deoxy-D-ribose, a polydeoxynucleotide containing D-ribose, an another type of polynucleotide which is N-glycoside of pyrine or pyrimidine base, or an another polymer having a non-nucleotide skeleton (for example, a commercially available protein nucleic acid and a synthetic sequence-specific nucleic acid polymer) or an another polymer containing a special linkage (provided that the polymer  
10 contains a nucleotide having arrangement permitting a phase pairing or base attachment found in a DNA or a RNA). They can be a double-stranded DNA, a single-stranded DNA, a double-stranded RNA, a single-stranded RNA, or a DNA:RNA hybrid, further may be an unmodified polynucleotide or an unmodified oligonucleotide, or further may have the known modification added, for example, may  
15 have a label which is known in the art, may have a cap added, may be methylated, may have 1 or more natural nucleotides substituted with an analogue, may have an intramolecular nucleotide modified, for example, may have non-charged linkage (for example, methylphosphonate, phosphotriester, phosphoramidate, carbamate and the like), may have a linkage having a charge or a sulfur-containing linkage (for example,  
20 phosphorothioate, phosphorodithioate and the like), for example, may have a side group such as a protein (nuclease, nuclease inhibitor, toxin, antibody, signal peptide, poly-L-lysine and the like) or a sugar (for example, monosaccharide and the like), may have an interchalant compound (for example, acridine, psoralen and the like), may have a chelating compound (for example, a metal, a metal having radioactivity, boron,  
25 an oxidative metal and the like), may have an alkylating agent, may have a modified

linkage (for example,  $\alpha$  anomer type nucleic acid). Here, "nucleoside", "nucleotide" and "nucleic acid" may not only contain a pyrine and a pyrimidine base but also another heterocyclic base. Such the modification may contain a methylated purine and pyrimidine, an acylated purine and pyrimidine, or an another heterocycle. In a 5 modified nucleoside and a modified nucleotide, a sugar part may be modified and, for example, 1 or more hydroxy groups may be substituted with a halogen or an aliphatic group, or may be converted into a functional group such as ether and amine.

An antisense nucleic acid of the present invention is a RNA, a DNA, or a modified nucleic acid. Embodiments of a modified nucleic acid are not limited to 10 but include a sulfur derivative or a thiophosphate derivative of a nucleic acid, and a nucleic acid having resistance to degradation of polynucleosideamide or oligonucleosideamide. An antisense nucleic acid of the present invention can be 15 preferably designed according to the following guideline. That is, an antisense nucleic acid is made stable in a cell, cell permeability of an antisense nucleic acid is enhanced, affinity for a target sense chain is made greater and, if it has toxicity, toxicity is made smaller.

A number of such the modifications are known in the art and are disclosed in J. Kawakami et al., Pharm Tech Japan, Vol. 8, pp.247 1992; Vol. 8, pp.395, 1992; S.T. Crooke et al. ed., Antisense Research and Applications, CRC Press, 1993.

20 An antisense nucleic acid of the present invention may be altered or may have a modified sugar, base or linkage, and can be supplied as a special form such as a microsphere, or may be applied to gene therapy, or may be given as an added form. As an antisense nucleic acid which is used as such an added form, there are a 25 polycation such as polylysine which exerts so as to neutralize a charge of a phosphate skeleton, and a hydrophobic lipid (for example, phospholipid, cholesterol and the like)

which increases uptake of a nucleic acid. As a preferable lipid to be added, there are cholesterol and its derivatives (for example, cholestryl chloroformate, cholic acid and the like). They can be attached to a 3'terminal or a 5'terminal of a nucleic acid, can be attached via a base, a sugar or an intramolecular nucleoside linkage. As the 5 other group, there is a group for capping which is specifically arranged at a 3'terminal or a 5'terminal of a nucleic acid and is for inhibiting degradation by nuclease such as exonuclease, RNase and the like. Examples of such the group for capping are not limited to but include protecting groups known in the art including glycols such as polyethylene glycol, tetraethylene glycol and the like.

10 The inhibitory activity of an antisense nucleic acid of the present invention can be examined using a transformant of the present invention, gene expressing system in and outside the living body of the present invention, or translation system for a protein in and outside the living body. The nucleic acid can be applied to cells by various method known per se.

15 A DNA encoding a partial peptide of the present invention may be any one as long as it has a nucleotide sequence encoding a partial peptide of the present invention. Further, the DNA may be any of a genomic DNA, a genomic DNA library, a cDNA derived from the aforementioned cells or tissues, a cDNA library derived from the aforementioned cells or tissues, or a synthetic DNA. A vector used for a library may 20 be any of bacteriophage, plasmid, cosmid, and phagemide. In addition, a mRNA fraction is prepared from the aforementioned cells or tissues, which may be used to directly amplify by Reverse Transcriptase Polymerase Chain Reaction (hereinafter, abbreviated as RT-PCR method).

More particularly, as a DNA encoding a partial peptide of the present  
25 invention may be, for example, a DNA having a partial nucleotide sequence of a DNA

which has a nucleotide sequence represented by SEQ ID No.:2, SEQ ID No.:4 or SEQ ID No.:6, or any DNA having a nucleotide sequence which hybridizes with a nucleotide sequence represented by SEQ ID No.:2, SEQ ID No.:4 or SEQ ID No.:6 under the high stringent conditions and having a partial nucleotide sequence of a DNA 5 encoding a protein having substantially the same nature of activity (for example, ligand binding activity, signal information transmitting action and the like) as that of a protein peptide of the present invention is used.

As a DNA which can hybridize with a nucleotide sequence represented by SEQ ID No.:2, SEQ ID No.:4 or SEQ ID No.:6, a DNA having a nucleotide sequence 10 having about 70% or more, preferably about 80% or more, more preferably about 90% or more, most preferably about 95% or more homology with a nucleotide sequence represented by SEQ ID No.:2, SEQ ID No.:4 or SEQ ID No.:6 is used.

For cloning a DNA fully encoding present protein or a partial peptide thereof (hereinafter, abbreviated as present protein), the DNA can be amplified by a PCR 15 method using a synthetic DNA primer having a partial nucleotide sequence of present protein, or can be selected by hybridization of a DNA incorporated into a suitable vector with a labeled DNA fragment or synthetic DNA encoding a part of or an entire region of present protein. A hybridization method may be performed by a method described in, for example, Molecular Cloning 2nd (J. Sambrook et al., Cold Spring 20 Harbor Lab. Press, 1989). In addition, when a commercially available library is used, it can be performed according to a method described in the attached specification.

Conversion of a nucleotide sequence of a DNA can be performed by the method known per se such as a Gapped duplex method or a Kunkel method or a similar method using Mutant<sup>TM</sup>-G (Takara shuzo Co., Ltd.), Mutant<sup>TM</sup>-K(Takara 25 shuzo Co., Ltd.) or the like.

A DNA encoding a cloned protein can be used as it is, or used optionally by digesting with a restriction enzyme, or adding a linker, depending upon the purposes. The DNA may have ATG as a translation initiation codon at its 5'terminal, or TAA, TGA or TAG as a translation termination codon at its 3'terminal. These termination initiation codon and translation termination codon can be added using a suitable synthetic DNA adapter.

An expression vector for present protein can be prepared by, for example, (a) excising a DNA fragment of interest from a DNA encoding present protein, (b) ligating the DNA fragment to downstream of a promoter in a suitable expression vector.

As a vector, a plasmid derived from Escherichia coli (for example, pBR322, pBR325, pUC12, pUC13), a plasmid derived from Bacillus subtilis (for example, pUB110, pTP5, pC194), a plasmid derived from yeast (for example, pSH19, pSH15), bacteriophage such as  $\gamma$  phase, animal virus such as retrovirus, vaccinia virus, baculovirus, as well as pA1-11, pXT1, pRc/CMV, pRc/RSV, pcDNAI/Neo and the like are used.

As a promoter used in the present invention, any promoter which is suitable for a host used for expressing a gene may be used. For example, when an animal cell is used as a host, there are SR $\alpha$  promoter, SV40 promoter, LTR promoter, CMV promoter, HSV-TK promoter and the like.

Among them, the use of CMV promoter and SR $\alpha$  promoter is preferable. When a host is a bacterium belonging to genus Escherichia, trp promoter, lac promoter, recA promoter,  $\lambda$ PL promoter, lpp promoter and the like are preferable and, when a host is a bacterium belonging to genus Bacillus, SPO1 promoter, SPO2 promoter, penP promoter are preferable and, when a host is yeast, PHO5 promoter,

PGK promoter, GAP promoter, ADH promoter and the like are preferable. When a host is an insect cell, polyhedron, P10 promoter and the like are preferable.

As an expression vector, a vector optionally containing enhancer, splicing signal, polyA addition signal, selectable marker, SV40 replication origin (hereinafter abbreviated as SV40ori in some cases) or the like may be used. As a selectable marker, for example, there are dihydrofolate reductase (hereinafter, abbreviated as dhfr in some cases) gene [methotrexate (MTX) resistant], ampicillin resistant gene (hereinafter, abbreviated as Amp<sup>r</sup> in some cases), neomycin resistant gene (hereinafter, abbreviated as Neo in some cases, G418 resistant) and the like. In particular, when dhfr gene is used as a selectable marker using CHO (dhfr<sup>-</sup>) cell, a gene of interest can be selected also on a medium containing no thymidine.

In addition, as necessary, a signal sequence compatible for a host is added to a N-terminal of present protein. When a host is a bacterium belonging to genus Escherichia, PhoA·signal sequence, Omp A·signal sequence and the like can be utilized and, when a host is a bacterium belonging to genus Bacillus,  $\alpha$ -amylase·signal sequence, subtilisin·signal sequence and the like can be utilized and, when a host is yeast, MF $\alpha$ ·signal sequence, SUC2·signal sequence and the like can be utilized and, when a host is an animal cell, insulin·signal sequence,  $\alpha$ -interferon·signal sequence, antibody·signal sequence and the like can be utilized.

A vector containing a DNA encoding present protein thus constructed can be used to produce a transformant.

As a host, for example, a bacterium belonging to genus Escherichia, a bacterium belonging to genus Bacillus, yeast, insect cell, insect, animal cell and the like are used.

As an example of a bacterium belonging to genus Escherichia, Escherichia

coli K12·DH1 [Proc. Natl. Acad. Sci. USA), vol.60, 160 (1968), JM103 [Nucleic Acids Research), Vol.9, 309(1981), JA211 [Journal of Molecular Biology], vol.120, 517 (1978), HB101[Journal of Molecular Biology, vol.41, 459(1969)], C600 [Genetics, vol.39, 440(1954)] and the like are used.

5 As an example of a bacterium belonging to genus Bacillus, for example, Bacillus subtilis MI114 [Gene, vol.24, 255 (1983)], 207-21 [Journal of Biochemistry, vol.95, 87(1984)] and the like are used.

As yeast, for example, Saccharomyces cerevisiae) AH22, AH22R<sup>+</sup>, NA87-11A, DKD-5D, 20B-12, Schizosaccharomyces pombe) NCYC1913, NCYC2036, Pichia 10 pastoris and the like are used.

As an insect cell, established cell derived from Barathra larva (Spodoptera frugiperda cell;Sf cell), MG1 cell derived from midgut of Trichoplusia ni, High Five<sup>TM</sup> cell derived from egg of Trichoplusia ni, cell derived from Mamestra brassicae, cell derived from Estigmene acrea and the like are used. When a virus is BmNPV, 15 established cell derived from silkworm (Bombyx mori N;BmN cell) and the like are used. As the Sf cell, for example, Sf9 cell (ATCC CRL1711), Sf21 cell (Vaughn, J.L. et al., In Vivo, 13, 213-217 (1977)) and the like are used.

As an insect, for example, silkworm larva and the like are used [Maeda et al., Nature, vol.315, 592(1985)].

20 As an animal cell, for example, monkey COS-7, Vero, Chinese hamster cell CHO (hereinafter, abbreviated as CHO cell), dhfr gene-deficient Chinese hamster cell CHO (hereinafter, abbreviated as CHO (dhfr<sup>-</sup>) cell), mouse L cell, mouse AtT-20, mouse myeloma cell, rat GH3, human FL cell and the like are used.

In order to transform a bacterium belonging to genus Escherichia, for 25 example, transformation can be performed according to a method described in Proc.

Natl. Acad. Sci. USA, vol.69, 2110(1972) and Gene, vol.17, 107(1982). In order to transform a bacterium belonging to genus Bacillus, for example, transformation can be performed according to a method described in Molecular & General Genetics, vol.168, 111(1979).

5 In order to transform yeast, transformation can be performed according to a method described, for example, in Methods in Enzymology, vol.194, 182-187 (1991), Proc. Natl. Acad. Sci. USA, vol.75, 1929(1978) and the like.

In order to transform an insect cell or an insect, transformation can be performed according to a method described in Bio/Technology, 6, 47-55(1988) and  
10 the like.

In order to transform an animal cell, transformation can be performed according to a method described, for example, in Cell Technology, separate column 8, New Cell Technology Experiment Protocol, 263-267 (1995) (published by Shujunsha), Virology, vol.52, 456(1973) and the like.

15 Like this, a transformant transformed with an expression vector containing a DNA encoding a G protein conjugated protein can be obtained.

When a transformant, a host of which is a bacterium belonging to genus Escherichia or a bacterium belonging to genus Bacillus, is cultured, as a medium used for culturing, a liquid medium is suitable and carbon source, nitrogen source,  
20 inorganic substances and other necessary for growth of the transformant are contained therein. As carbon source, for example, there are glucose, dextrin, soluble starch, sucrose and the like. As nitrogen source, for example, there are inorganic and organic substances such as ammonium salts, nitrate salts, corn steep liquor, peptone, casein, broth extract, soy bean bran, potato extract solution and the like. As an inorganic substance, for example, there are calcium chloride, sodium dihydrogen

phosphate, magnesium chloride and the like. In addition, yeast, vitamines, growth promoting factor and the like may be added. pH of a medium is desirably about 5-8.

As a medium when a bacterium belonging to Escherichia is cultured, for example, the M9 medium containing glucose and casamino acid [Miller, Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York, 1972] is preferable. In order to allow a promoter to exert effectively as necessary, for example, a chemical such as 3 $\beta$ -indolyl acrylic acid can be added.

When a host is a bacterium belonging to genus Escherichia, culturing is usually performed at about 15-43°C for about 3-24 hours and, if needed, airation and stirring may be added.

When a host is a bacterium belonging to genus Bacillus, culturing is usually carried out at about 30-40°C for about 6-24 hours and airation and stirring may be added as necessary.

When a transformant, a host of which is yeast, is cultured, as a medium, there are Burkholder minimum medium [Bostian, K.L. et al. (Proc. Natl. Acad. Sci. USA, vol.77, 4505(1980)] and the SD medium containing 0.5% casamino acid [Bitter, G.A. et al. (Proc. Natl. Acad. Sci. USA), vol.81, 5330(1984)]. pH of a medium is preferably adjusted to about 5-8. Culturing is usually performed at about 20-35°C for about 24-72 hours and airation and stirring are added as necessary.

When a transformant, a host of which is an insect cell or an insect, is cultured, as a medium, Grace's Insect Medium (Grace T.C.C., Nature, 195, 788 (1962)) to which an additive such as immobilized 10% bovine serum is appropriately added and the like are used. pH of a medium is preferably adjusted to about 6.2-6.4. Culturing is usually performed at about 27°C for 3-5 days and airation and stirring are added as necessary.

When a transformant, a host of which is an animal cell, is cultured, as a medium, MEM medium containing about 5-20% fetal bovine serum [Science, vol.122, 501 (1952)], DMEM medium [Virology, vol.8, 396(1959)], RPMI 1640 medium [The Journal of the American Medical Association, vol.199, 519(1967)], 199 medium

- 5 [Proceedings of the Society for the Biological Medicine, vol.73, 1(1950)] and the like are used. pH is preferably about 6-8. Culturing is performed at about 30-40°C for 15-60 hours and airation and stirring are added as necessary.

Like this, a G protein coupled receptor protein of the present invention can be produced in a cell membrane of a transformant.

- 10 In order to purify and isolate present protein from the aforementioned culture, for example, purification and isolation can be performed according to the following method.

Upon extraction of present protein from cultured bacterium or cells, a method of collecting the bacterium or cell by the known method after culturing, suspending 15 this in a suitable buffer, disrupting the bacterium or cell by lysozyme and/or freeze-melting, and obtaining a crude extract of a protein by centrifugation or filtration or the like is appropriately used. A protein denaturing agent such as urea and guanidine hydrochloride, or a surfactant such as TritonX-100<sup>TM</sup> may be contained in the buffer.

- 20 When a protein is secreted in the culturing solution, after culturing is complete, the bacterium or cell and the supernatant are separated by the method known per se and the supernatant is collected.

Purification of the culturing supernatant thus obtained or a protein contained in the extract may be performed by combining the separating and purifying methods known per se. As the known separating and purifying methods, a method utilizing 25 the solubility such as salting out and a solvent precipitation method, a method

utilizing mainly a difference in a molecular weight such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide electrophoresis, a method utilizing a difference in charge such as ion-exchange chromatography, a method utilizing the specific affinity such as affinity chromatography, a method utilizing a difference in hydrophobicity 5 such as reverse phase high performance liquid chromatography, a method utilizing a difference in isoelectric point such as electrofocusing and the like are used.

When the protein thus obtained is obtained as a free material, it can be converted into a salt by the method known per se or a similar method and, conversely, when the protein is obtained as a salt, it can be converted into a free material or other 10 salt by the method known per se or a similar method.

By acting a suitable protein modifying enzyme before or after purification, a protein produced by a transformant may be modified or a polypeptide may be partially removed therefrom. As a protein modifying enzyme, for example, trypsin, chymotrypsin, aluginineendopeptidase, proteinkinase, glucosidase and the like are 15 used.

The activity of present protein or a salt thereof thus obtained can be measured by an experiment for binding with a labeled ligand, enzyme immunoassay and the like.

An antibody to present protein, a partial peptide thereof or a salt thereof may be any of polyclonal antibody and monoclonal antibody as long as it is an antibody 20 which can recognize present protein, a partial peptide thereof or a salt thereof.

An antibody to present protein, a partial peptide thereof or a salt thereof (hereinafter, abbreviated as present protein or the like) can be prepared using the present protein or the like as an antigen according to the method for preparing an antibody or antiserum known per se.

[Preparation of a monoclonal antibody]

(a) Preparation of a monoclonal antibody producing cell

The present protein or the like is administered to a site of a mammal being capable of producing an antibody as it is or together with a carrier or a diluent. In 5 order to enhance the antibody producing ability upon administration, Freund's complete adjuvant or Freund's incomplete adjuvant may be administered.

Administration is usually conducted once per 2-6 weeks at a total of around 2-10 times. As a mammal to be used, although there are, for example, monkey, rabbit, dog, guinea pig, mouse, rat, sheep and goat, mouse and rat are preferably used.

10 Upon preparation of a monoclonal antibody producing cell, a monoclonal producing hybridoma can be prepared by selecting a warm blood animal immunized with an antigen, for example, an individual having recognized titer from mice, taking spleen or lymph node 2-5 days after final immunization, and fusing an antibody producing cell contained therein with a myeloma cell. Measurement of the antibody 15 titer in antiserum can be performed, for example, by measuring the activity of a labeling agent bound to an antibody after the labeled present protein or the like described below is reacted with antiserum. The fusing operation can be performed, for example, by a method of Khler and Milstein [Nature, vol.256, pp.495 (1975)]. As a fusion promoting agent, for example, although there are polyethylene glycol 20 (PEG), Sendai virus and the like, PEG is preferably used.

As a myeloma cell, although there are, for example, NS-1, P3U1, SP2/0 and the like, P3U1 is preferably used. A preferable ratio of the number of antibody producing cells (spleen cells) used and that of myeloma cells used is around 1:1-20:1. PEG (preferably PEG 1000-PEG 6000) is added at the concentration of around 10-25 80%. Cell fusion can be performed effectively by incubation at about 20-40°C,

preferably about 30-37°C for about 1-10 minutes.

For screening a monoclonal antibody producing hybridoma, a variety of methods can be used. For example, there are a method of adding the hybridoma culturing supernatant to a solid phase on which the present protein or the like is adsorbed directly or together with a carrier (for example, microplate) and, then, adding an anti-immunoglobulin antibody (when a cell used in cell fusion is mouse, an anti-mouse immunoglobulin antibody is used) labeled with a radioisotope or an enzyme or protein A to detect a monoclonal antibody bound to a solid phase, a method of adding the hybridoma culturing supernatant to a solid phase on which anti-immunoglobulin antibody or a protein A is adsorbed and adding the present protein or the like labeled with a radioisotope or an enzyme to detect a monoclonal antibody bound to a solid phase and the like.

Although selection of a monoclonal antibody can be performed according to the method known per se or a similar method, it can be usually performed with a medium for an animal cell to which HAT (hypoxanthine, aminopterin, thymidine) is added. As a medium for selection and growth, any medium may be used as long as a hybridoma can be grown therein. For example, RPMI 1640 medium containing 1-20%, preferably 10-20% fetal bovine serum, GIT medium containing 1-10% fetal bovine serum (Wako Pure Chemical Industries, Ltd.) or serum-free medium for growing a hybridoma (SFM-101, Nissui Pharmaceutical Co., Ltd.) and the like can be used. A culturing temperature is usually 20-40°C, preferably about 37°C. A culturing time is usually 5 days to 3 weeks, preferably 1 to 2 weeks. Culturing can be usually performed under 5% carbonic acid gas. The antibody titer of the hybridoma culturing supernatant can be measured as in the measurement of the antibody titer of antiserum described above.

(b) Purification of a monoclonal antibody

Separation and purification of a monoclonal antibody can be usually performed according to a separation and purification method of an immunoglobulin [for example, salting out method, alcohol precipitation method, isoelectric focusing method, electrophoresis method, adsorption and desorption method by ion exchanging material (for example, DEAE), ultracentrifugation method, gel filtration method, a specific purifying method of taking only antibody with an active adsorbing agent such as antigen binding solid phase, protein A or protein G].

10

[Preparation of a polyclonal antibody]

The polyclonal antibody of the present invention can be prepared according to the method known per se or a similar method. For example, it can be prepared by making a complex of an immunological antigen (receptor protein and the like antigen) and a carrier protein, which is used to immunize a mammal as in the case of the aforementioned preparation of a monoclonal antibody, and taking a material containing an antibody to the present protein or the like from the immunized mammal to perform separation and purification of an antibody.

As a complex of an immunoantigen and a carrier protein which is used for immunizing a mammal, any of a kind of a carrier protein and a mixturing ratio of a carrier and a hapten may be used for cross-linking as long as an antibody can be effective to a hapten immunized by cross-linking with a carrier. For example, a method for coupling bovine serum alubmin, bovine thyroglobulin, keyhole limpet hemocyanin and the like with a hapten at a rate by weight of about 0.1-20, preferably about 1-5 relative to 1 of hapten is used.

In addition, for coupling hapten with a carrier, a variety of condensing agents can be used, and glutaraldehyde and carbodiimide, maleimide-active ester, active ester reagent containing a thiol group or a dithiobryidyl group can be used.

A condensation product can be administered to a site of a mammal which can produce an antibody as it is or together with a carrier or a diluent. In order to enhance the antibody producing ability upon administration, Freund's complete adjuvant or Freund's incomplete adjuvant can be administered. Administration can be usually performed once per about 2-6 weeks, at a total about 3-10 times.

A polyclonal antibody can be taken from blood, ascites and the like, preferably blood of a mammal immunized according to the above method.

The polyclonal titer in antiserum can be measured as in the aforementioned measurement of the antibody titer in serum. Separation and purification of a polyclonal antibody can be carried out according to separation and purification of immunoglobulin as in the aforementioned separation and purification of a monoclonal antibody.

Present protein, a partial peptide thereof or a salt thereof can be used as (1) a method of determining a ligand for present protein, (2) obtaining of an antibody and antiserum, (3) construction of an expression system for a recombinant protein, (4) development of a receptor binding assay using the same expression system, and screening of medicine candidates, (5) implementation of drug design based on comparison with a ligand receptor having the structural similarity, (6) a reagent for making a probe and a PCR primer in gene therapy, (7) production of a transgenic animal, or (8) a drug for gene prophylaxis and therapy.

In particular, a compound which alters property of a ligand binding with a G protein-coupled receptor specific for human being or a mammal (for example, agonist,

antagonist and the like) can be screened by using a receptor binding assay system using an expression system for a recombinant protein of the present invention, and the agonist and the antagonist can be used as an agent for preventing or treating various diseases.

5       The use of present protein, a partial peptide or a salt thereof (hereinafter, abbreviated as present protein or the like in some cases), a DNA encoding present protein or a partial peptide thereof (hereinafter, abbreviated as present DNA in some cases) and an antibody to the present invention or the like (hereinafter, abbreviated as present antibody in some cases) is explained more particularly.

10           (1) A method of determining a ligand (agonist) for present protein

Present protein or a salt thereof or a partial peptide of the present invention or a salt thereof are useful as a reagent for searching or determining a ligand (agonist) for present protein or a salt.

15           That is, the present invention provides a method for determining a ligand for present protein, which comprises contacting a test compound with present protein or a salt thereof or a partial peptide of the present invention or a salt thereof.

As a test compound, there are the known ligand (for example, angiotensin, bombesin, cannabinoid, cholecystokinin, glutamine, serotonin, melatonin, neuropeptide Y, opioid, purine, vasopressin, oxytocin, PACAP, secretin, glucagon, calcitonin, adrenomedulin, somatostatin, GHRH, CRF, ACTH, GRP, PTH, VIP (vasoactive intestinal and related polypeptide), somatostatin, dopamine, motilin, amylin, bradykinin, CGRP (calcitonin gene related peptide), leukotriene, pancreastatin, prostaglandin, thromboxane, adenosine, adrenaline,  $\alpha$  and  $\beta$ -chemokine (for example, IL-8, GRO $\alpha$ , GRO $\beta$ , GRO $\gamma$ , NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14,

MCP-3, I-309, MIP1 $\alpha$ , MIP-1 $\beta$ , RANTES and the like), endothelin, enterogastrin, histamine, neuropeptides, neurotensin, TRH, pancreatic polypeptide, galanin), subtype thereof or analogue thereof, known  $\alpha$ -latrotoxin, neurexophilin, subtype thereof, or analogue thereof, and for example, tissues extract, and cell culture supernatant of human being  
5 or a mammal (for example, mouse, rat, pig, cow, sheep, monkey and the like) are used. For example, the tissue extract, cell culture supernatant or the like is added to present protein, fractionated while measuring the cell stimulating activity and, finally, a single ligand can be obtained. Inter alia,  $\alpha$ -latrotoxin, neurophilin, subtype thereof or an analogue thereof are a preferable ligand.

10 More particularly, a method for determining a ligand of the present invention is a method for determining a compound (for example, peptide, protein, non-peptide compound, synthetic compound, fermented product and the like) or a salt thereof having the cell stimulating activity (for example, activity of promoting or inhibiting arachidonic acid release, acetylcholine release, intracellular  $\text{Ca}^{2+}$  release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, intracellular potential variation, phosphorylation of an intracellular protein, c-fos activation, decrease in pH and the like) by binding to present protein using present protein, a partial peptide thereof or a salt thereof, or using the receptor binding assay system using a constructed expression system for a recombinant protein.  
15

20 A method for determining a ligand of the present invention is characterized in that an amount of a test compound bound to present protein or a partial peptide thereof when a test compound is contacted with the protein or the partial peptide.

More particularly, the present invention provides:

- (1) a method for determining the ligand for present protein or a salt thereof,  
25 which comprises measuring an amount of a labeled test compound bound to present

protein or a salt thereof or a partial peptide of the present invention or a salt thereof when a labeled compound is contacted with the protein or the salt or the partial peptide or the salt,

(2) a method for determining a ligand for present protein or a salt thereof,  
5 which comprises measuring an amount of a labeled test compound bound to a cell containing present protein or a membrane fraction of a cell when a labeled test compound is contacted with the cell or the membrane fraction,

(3) a method for determining a ligand for present protein, which comprises  
measuring an amount of a labeled test compound bound to a protein expressed on a  
10 cell membrane by culturing a transformant containing a DNA encoding present protein when a labeled test compound is contacted with the protein or a salt thereof,

(4) a method for determining a ligand for present protein or a salt thereof,  
which comprises measuring the cell stimulation activity (for example, the activity of  
promoting or inhibiting arachidonic acid release, acetylcholine release, intracellular  
15  $\text{Ca}^{2+}$  release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, cell membrane potential variation, phosphorylation of  
intracellular protein, activation of c-fos, decrease in pH and the like) via a protein  
when a test compound is contacted with a cell containing present protein,

(5) a method for determining a ligand for present protein or a salt thereof,  
20 which comprises measuring the cell stimulation activity (for example, the activity of  
promoting or inhibiting arachidonic acid release, acetylcholine release, intracellular  
 $\text{Ca}^{2+}$  release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, cell membrane potential variation, phosphorylation of  
intracellular protein, activation of c-fos, decrease in pH and the like) via a protein  
25 when a test compound is contacted with a protein expressed on a cell membrane by

culturing a transformant containing a DNA encoding present protein.

In particular, it is preferable that, after the aforementioned tests (1)-(3) are conducted and the binding of a test compound with present protein is confirmed, the aforementioned (4)-(5) are conducted.

5 First, although a protein used for a method for determining a ligand may be any protein as long as it contains the aforementioned protein of the present invention or the partial peptide of the present invention, a protein which is expressed using an animal cell is suitable.

In order to prepare present protein, the aforementioned expression method is  
10 used and it is preferable that preparation is conducted by expressing a DNA encoding the protein in a mammal cell or an insect cell. As a DNA fragment encoding a protein part of interest, a complementary DNA is usually used and the fragment is not necessarily limited to it. For example, a gene fragment and a synthetic DNA may be used. In order to introduce a DNA fragment encoding present protein into a host  
15 animal cell and express it effectively, it is preferable that the DNA fragment is incorporated downstream of polyhedron promoter of nuclear polyhedrosis virus (NPV) belonging to genus baculovirus, promoter derived from SV40, promoter of retrovirus, metallothionein promoter, human heat shock promoter, cytomegalovirus promoter, SR $\alpha$  promoter and the like. Examination of an amount and quality of  
20 expressed receptor can be conducted by the method known per se. For example, examination can be conducted according to a method described in the literature [Nambi, P. et al., The Journal of Biological Chemistry, J. Biol. Chem., vol.267, 19555-19559, 1992].

Therefore, as one containing present protein, a partial peptide thereof or a salt  
25 thereof, it may be a protein purified according to the method known per se, a partial

peptide thereof or a salt thereof, or a cell containing the protein or its cell membrane fraction may be used.

In a method for determining a ligand, when a cell containing present protein is used, the cell may be fixed by glutaraldehyde, formalin or the like. A fixing 5 method can be conducted according to the method known per se.

A cell containing present protein refers to a host cell which expressed present protein. As the host cell, Escherichia coli, Bacillus subtilis, yeast, insect cell, animal cell and the like are used.

A cell membrane fraction refers to a fraction in which a cell membrane 10 obtained by the method known per se after a cell is ruptured is contained at a large amount. As a method for rupturing a cell, there are a method of squeezing a cell with a Potter-Elvehjem type homogenizer, rupture with Waring blender or Polytron (manufactured by Kinematica), rupture by ultrasound, rupture by ejecting a cell through a thin nozzle while pressing with a French press and the like. For 15 fractionating a cell membrane, a fractionating method by centrifugation such as a fractionation centrifugation method and a density gradient centrifugation method are mainly used. For example, a ruptured cell solution is centrifuged at a low speed (500 rpm-3000 rpm) for a shorter period of time (usually, about 1 minute to 10 minutes), a supernatant is further centrifuged at a higher speed (15000 rpm-30000 rpm) usually 20 for 30 minutes to 2 hours and the resulting precipitates are used as a membrane fraction. An expressed protein and a membrane component such as phospholipid and membrane protein derived from a cell are contained in the membrane fraction at a large amount.

An amount of a cell containing the protein or a protein in the membrane 25 fraction is preferably  $10^3$ - $10^8$  molecules per 1 cell, suitably  $10^5$ - $10^7$  molecules. As

an expression amount grows larger, the ligand binding activity (specific activity) is increased and, thereby, not only construction of high sensitive screening system becomes possible but also a large amount of samples can be measured at the same lot.

In order to perform the aforementioned methods (1)-(3) for determining a

- 5 ligand for present protein or a salt thereof, a suitable protein fraction and a labeled test compound are necessary.

As a protein fraction, a natural receptor protein fraction or a recombinant receptor fraction having the equivalent activity thereto are desirable. Here, equivalent activity denotes equivalent ligand binding activity, signal information 10 transmitting action and the like.

As a labeled test compound, angiotensin, bombesin, cannabinoid, cholecystokinin, glutamine, serotonin, melatonin, neuropeptide Y, opioid, purine, vasopressin, oxytocin, PACAP, secretin, glucagon, calcitonin, adrenomedulin, somatostatin, GHRH, CRF, ACTH, GRP, PTH, VIP (vasoactive intestinal and related 15 polypeptide), somatostatin, dopamine, motilin, amylin, bradykinin, CGRP (calcitonin gene related peptide), leukotriene, pancreastatin, prostaglandin, thromboxane, adenosine, adrenaline,  $\alpha$  and  $\beta$ -chemokine (for example, IL-8, GRO $\alpha$ , GRO $\beta$ , GRO $\gamma$ , NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, MCP-3, I-309, MIP1 $\alpha$ , MIP-1 $\beta$ , RANTES and the like), endothelin, enterogastrin, histamine, neurotensin, TRH, 20 pancreatic polypeptide, galanin,  $\alpha$ -latrotoxin, neurexophilin, subtype thereof, or analogue thereof labeled with [ $^3$ H], [ $^{125}$ I], [ $^{14}$ C], [ $^{35}$ S] or the like are suitable.

Among them,  $\alpha$ -latrotoxin, neuroxophilin, subtype thereof or an analogue thereof are preferably used.

More particularly, in order to perform a method for determining a ligand for

- 25 present protein or a salt thereof, an authentic receptor is first prepared by suspending a

cell containing present protein or a membrane fraction of the cell in a buffer suitable for determination. As a buffer, any buffer such as phosphate buffer having pH 4-10 (desirably, pH6-8) and Tris-HCl buffer may be used as long as it dose not inhibit binding of a ligand with present protein. In addition, in order to reduce the non-specific binding, surfactants such as CHAPS, Tween-80<sup>TM</sup> (Kao-Atlas), digitonin and deoxycholate, and proteins such as serum albumin and gelatin may be added to a buffer. Further, in order to suppress degradation of lipase or a ligand by protease, protease inhibiting agents such as PMSF, leupeptin, E-64 (manufactured by Peptide Laboratory) and pepstatin may be added thereto. A test compound labeled with [<sup>3</sup>H], [<sup>125</sup>I], [<sup>14</sup>C], [<sup>35</sup>S] or the like at a constant amount (5000 cpm-500000 cpm) is present in 0.01 ml-10 ml of a solution of the receptor. In order to know an amount of non-specific binding (NBS), a reaction tube to which a large excess unlabeled test compound is added is prepared. A reaction is performed at 0°C to 50°C, desirably at about 4°C to 37°C for about 20 minutes to 24 hours, desirably for about 30 minutes to 3 hours. After reaction, the reaction is filtered with a glass fiber filter or the like, washed with a suitable amount of a buffer, and the radioactivity remaining on a glass fiber filter is measured with a liquid scintillation counter or a  $\gamma$ -counter. A compound having the count (non-specific binding amount (NSB) is subtracted from whole binding amount (B)) (B-NSB) over 0 cpm can be a ligand (an agonist) for present protein or a salt thereof.

In order to perform the aforementioned methods (4)-(5) for determining a ligand for present protein or a salt thereof, the cell stimulating activity (for example, activity of promoting or inhibiting arachidonic acid release, acetylcholine release, intracellular  $\text{Ca}^{2+}$  release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, intracellular potential variation,

phosphorylation of an intracellular protein, c-fos activation, decrease in pH and the like) via the protein can be measured using the known protein or a commercially available measuring kit. More particularly, first, a cell containing present protein is cultured on a multiwell-plate or the like. Upon implementation of ligand

5 determination, a test compound and the like are added, incubated for a certain time, the cell is extracted or the supernatant is recovered to quantitate the resulting products according to respective methods. When the production of a substance [for example, arachidonic acid and the like] which is an index for the cell stimulating activity is difficult to be detected due to degradation of a degrading enzyme contained in the cell, 10 an inhibiting agent for the degrading enzyme may be added to perform an assay. In addition, regarding the activity of cAMP production suppression, it can be detected as production inhibiting action for a cell for which fundamental producing amount has been increased with forskolin.

A kit for determining a ligand for present protein or a salt thereof contains 15 present protein or a salt thereof, a partial peptide of the present invention or a salt thereof, a cell containing present protein, or a membrane fraction of a cell containing present protein.

As an example of a kit for determining a ligand of the present invention, there are the following ones:

20

1. A reagent for determining a ligand

(1) A buffer for measurement and a buffer for washing

0.05% bovine serum albumin (manufactured by Sigma) is added to Hank's Balanced Salt Solution (manufactured by Gibco)

25 Filter-sterilized with a filter having a pore size 0.45 µm, which may be stored

at 4°C or prepared upon use.

(2) An authentic G protein-coupled receptor protein

CHO cell in which present protein is expressed is subcultured on a 12-well plate at  $5 \times 10^5$  cells/well, and cultured at 37°C, in 5% CO<sub>2</sub> and 95% air.

5 (3) A labeled test compound

A compound labeled with commercially available [<sup>3</sup>H], [<sup>125</sup>I], [<sup>14</sup>C], [<sup>35</sup>S] or the like, or labeled by a suitable method.

An aqueous solution is stored at 4°C or -20°C and diluted with a measuring buffer upon use. A test compound which shows the poor water-solubility is dissolved in dimethylformamide, DMSO, methanol or the like.

10 (4) Non-labeled test compound

Prepared to 100-1000 fold higher concentration as in a labeled compound.

2. A detecting method

15 (1) CHO cell, expressing present protein, which was cultured on a 12-well plate for tissue culturing is washed twice with a 1 ml of a measuring buffer, and a 490 µl of a measuring buffer is added to each well.

20 (2) 5 µl of a labeled test compound is added, which is reacted at room temperature for 1 hour. In order to known an amount of non-specific binding, 5 µl of a non-labeled test compound is added.

(3) The reaction solution is removed and washed with 1 ml of a washing buffer three times. A labeled test compound bound to a cell is dissolved with 0.2N NaOH-1% SDS, and mixed with 4 ml of liquid scintillator A (manufactured by Wako Pure Chemical Industries, Ltd.).

25 (4) The radioactivity is measured using a liquid scintillation counter

(manufactured by Beckmann).

As a ligand which can bind to present protein or a salt thereof, there are substances which are specifically present, for example, in brain, pituitary gland, pancreas or the like. More particularly, angiotensin, bombesin, cannabinoid, 5 cholecystokinin, glutamine, serotonin, melatonin, neuropeptide Y, opioid, purine, vasopressin, oxytocin, PACAP, secretin, glucagon, calcitonin, adrenomedulin, somatostatin, GHRH, CRF, ACTH, GRP, PTH, VIP (vasoactive intestinal and related polypeptide), somatostatin, dopamine, motilin, amylin, bradykinin, CGRP (calcitonin gene related peptide), leukotriene, pancreastatin, prostaglandin, thromboxane, 10 adenosine, adrenaline,  $\alpha$  and  $\beta$ -chemokine (for example, IL-8, GRO $\alpha$ , GRO $\beta$ , GRO $\gamma$ , NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, MCP-3, I-309, MIP1 $\alpha$ , MIP-1 $\beta$ , RANTES and the like), endothelin, enterogastrin, histamine, neurotensin, TRH, pancreatic polypeptide, galanin,  $\alpha$ -latrotoxin, neurexophilin, subtype thereof, or analogue thereof are used. Inter alia,  $\alpha$ -latrotoxin, neurexophilin, subtype thereof, or 15 analogue thereof are preferably used.

(2) An agent for preventing or treating present protein deficient-disease

In the aforementioned method (1), if a ligand for present protein is revealed, the present protein or a DNA encoding the present protein can be used as a medicine 20 such as an agent for preventing or treating present protein deficient-disease.

For example, regarding a patient in which physiological action of a ligand is not expected due to decrease in present protein in the living body, an amount of a protein in the living body of a patient can be increased and an action of a ligand can be sufficiently exerted by (1) administering present protein to a patient to supplement an 25 amount of the protein, or (2) (a) administering a DNA encoding present protein to the

patient to express it, or (b) inserting a DNA encoding present protein to express it, which is transplanted into the patient. Therefore, a DNA encoding present protein is useful as a safe and low-toxic medicine for preventing or treating present protein deficient-disease.

5 When a DNA encoding present protein (hereinafter, abbreviated as present DNA in some cases) is used as the aforementioned preventing or treating agent, present DNA can be used according to the conventional method as it is or by inserting into a suitable vector such as retrovirus vector, adenovirus vector, adenovirus associated virus vector or the like. Present DNA can be administrated as it is or  
10 together with an adjuvant for promoting uptake by a gene gun or a catheter such as a hydrocatheter.

For example, present DNA can be orally administrated as a tablet optionally coated with a sugar coating, capsule, elixir or microcapsule and the like, or parenterally administrated in the form of a sterile solution with water or other  
15 pharmaceutically acceptable solution or an injectable such as suspension. For example, pharmaceutical preparations can be made by mixing present DNA with a carrier, a flavor, an excipient, a vehicle, a preservative, a stabilizer or a binding agent into an unit dosage form required for art-recognized preparations. An amount of an ingredient in these preparations is adjusted such that a suitable dose can be obtained in  
20 a designated range.

As an additive which can be mixed into a tablet, a capsule or the like, for example, a binding agent such as gelatin, corn starch, tragacanth and gum arabic, an excipient such as crystalline cellulose, a swelling agent such as corn starch, gelatin and alginic acid, a lubricating agent such as magnesium stearate, a sweetening agent  
25 such as sucrose, lactose and saccharin, a flavor such as peppermint, an almond oil

and cherry are used. When a preparation unit form is a capsule, a liquid carrier such as a lipid can be contained in the aforementioned type material. A sterile composition for injection can be formulated according to the conventional pharmacy by such as dissolving or suspending an active substance in a vehicle such as water for injection, a natural vegetable oil such as a sesami oil and a palm oil. As an aqueous solution for injection, for example, an isotonic (for example, D-sorbitol, D-mannitol, sodium chloride or the like) containing, for example, a physiological saline, glucose or other adjuvant and the like are used, and may be used together with a suitable solubilizer, for example, an alcohol (for example, ethanol), a polyalcohol (for example, propylene glycol, polyethylene glycol), nonionic surfactant (for example, polysorbate 80 (TM), HCO-50) or the like. As an oily solution, for example, a sesame oil and a soybean oil are used, and may be used together with benzyl benzoate, benzylalcohol or the like.

In addition, the aforementioned preventing or treating agent may be incorporated with, for example, a buffer (for example, a phosphate buffer, a sodium acetate buffer), a soothing agent (for example, benzalkonium chloride, procaine hydrochloride and the like), a stabilizing agent (for example, human serum alubmin, polyethylene glycol and the like), a preservative (for example, benzylalcohol, phenol and the like), and an antioxidant. A prepared injectable is usually filled into a suitable ampul.

Since pharmaceutical preparations thus obtained are safe and low toxic, and can be administered to, for example, human being or a mammal (for example, rat, rabbit, sheep, pig, cow, cat, dog, monkey).

A dose of present protein or DNA is different depending upon an administration subject, a subject organ, symptom and an administration method and,

in the case of oral administration, the dose is generally about 0.1 mg-100 mg, preferably about 1.0-50 mg, more preferably about 1.0-20 mg per day, in an adult (60 kg). When parentally administrated, one time dose is different depending upon an administration subject, a subject organ, symptom and an administration method and, 5 in the form of injectable, it is usually advantageous to administer about 0.01-30 mg, preferably about 0.1-20 mg, more preferably about 0.1-10 mg by intravenous injection per day, in an adult (60 kg). In other animals, an amount calculated per 60 kg can be administrated.

10 (3) An agent for diagnosing a gene

Since abnormality (gene abnormality) of a DNA or a mRNA encoding present protein in human being or a mammal (for example, rat, rabbit, sheep, pig, cow, cat, dog, monkey and the like) can be detected by using present DNA as a probe, for example, the present DNA is useful as an agent for diagnosing a gene such as damage 15 of the DNA or the mRNA, mutation or decrease in expression, increase in excess expression of the DNA or the mRNA.

The aforementioned gene diagnosis using present DNA can be conducted by, for example, northern hybridization and a PCR-SSCP method which are known per se (Genomics, vol. 5, 874-879 (1989), Proceedings of the National Academy of Sciences 20 of the United States of America), vol.86, pp.2766-2770 (1989)).

(4) A method for quantitating a ligand for present protein

Since present protein or the like has the binding property to a ligand, the concentration of a ligand in the living body can be quantitated with high sensitivity.

25 A quantitating method of the present invention can be used by combining

with, for example, a competition method. That is, the concentration of a ligand in a specimen by contacting a specimen with present protein or the like. More particularly, for example, it can be used according to a method described in the following (i) or (ii) or a similar method.

5 (i) "Radioimmuno assay" edited by Hiroshi Irie (Kodansha, published in 1973)

(ii) "A Sequel to the Radioimmuno assay" edited by Hiroshi Irie (Kodansha, published in 1978)

10 10 (5) A method for screening a compound which alters property of ligand binding with present protein

By using the receptor binding assay by using present protein or the like, or by constructing an expression system for a recombinant protein and using the expression system, a compound which alter property of a ligand binding with present protein or 15 the like (for example, peptide, protein, non-peptide compound, synthetic compound, fermented product and the like) or a salt thereof can be effectively screened.

20 (a) a compound having the cell stimulating activity (for example, activity of promoting or suppressing arachidonic acid release, acetylcholin release, intracellular  $\text{Ca}^{2+}$  release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, cell membrane potential variation, phosphorylation of intracellular protein, activation of c-fos and decrease in pH) via a G protein-coupled receptor (so called agonist to present protein), (b) a compound having no such the cell stimulating activity (so called antagonist to present protein), (c) a compound which enhances binding force of a ligand with present protein, and (d) a compound which 25 decreases binding force of a ligand with present protein are contained in such the

compound (it is preferred that the above (a) compound is screened by the aforementioned method for determining a ligand).

That is, the present invention provides a method for screening a compound which alters property of a ligand with present protein, a partial peptide thereof or a salt thereof, which comprises comparing (i) the case where present protein, a partial peptide thereof or a salt thereof and a ligand are contacted, and (ii) the case where present protein, a partial peptide thereof or a salt thereof and a ligand and a test compound are contacted.

In a method for screening of the present invention is characterized in that, for example, an amount of a ligand to bind with the protein or the like and cell stimulating activity in the cases (i) and (ii) are measured and compared.

More particularly, the present invention provides:

(1) a method for screening a compound which alters property of a ligand binding with present protein or the like or a salt thereof, which comprises measuring and comparing an amount of a labeled ligand to bind to present protein or the like in the case where a labeled ligand is contacted with present protein or the like, and the case where a labeled ligand and a test compound are contacted with present protein or the like,

(2) a method for screening a compound which alters property of a ligand binding with present protein or the like or a salt thereof, which comprises measuring and comparing an amount of a labeled ligand to bind to a cell containing present protein or the like or a membrane fraction of the cell in the case where a labeled ligand is contacted with the cell or the membrane fraction, and the case where a labeled ligand and a test compound are contacted with the cell or the membrane fraction,

(3) a method for screening a compound which alters property of a ligand

binding with present protein or the like or a salt thereof, which comprises measuring and comparing an amount of a labeled ligand to bind to protein or the like expressed on a cell membrane by culturing a transformant containing present DNA in the case where a labeled ligand is contacted with the protein or the like, and the case where a

5 labeled ligand and a test compound are contacted with the protein or the like,

(4) a method for screening a compound which alters property of a ligand binding with present protein or the like, which comprises measuring and comparing cell stimulating activity (for example, activity of promoting or suppressing arachidonic acid release, acetylcholin release, intracellular  $\text{Ca}^{2+}$  release, intracellular

10 cAMP production, intracellular cGMP production, inositol phosphate production, cell membrane potential variation, phosphorylation of intracellular protein, activation of c-fos and decrease in pH) via a receptor in the case where a compound which activates present protein or the like (for example, ligand for present protein or the like) is contacted with a cell containing present protein or the like, and the case where a

15 compound which activates present protein or the like and a test compound are contacted with a cell containing present protein or the like, and

(5) a method for screening a compound which alters property of a ligand

binding with present protein or the like, which comprises measuring and comparing cell stimulating activity (for example, activity of promoting or suppressing arachidonic acid release, acetylcholin release, intracellular  $\text{Ca}^{2+}$  release, intracellular

20 cAMP production, intracellular cGMP production, inositol phosphate production, cell membrane potential variation, phosphorylation of intracellular protein, activation of c-fos and decrease in pH) via a receptor in the case where a compound which activates present protein or the like (for example, ligand for present protein or the like) is

25 contacted with present protein or the like expressed on a cell membrane by culturing a

transformant containing present DNA, and the case where a compound which activates present protein or the like and a test compound are contacted with present protein or the like expressed on a cell membrane by culturing a transformant containing present DNA.

5 Before present protein or the like is obtained, when a G protein-coupled receptor agonist or antagonist is screened, a test was necessary in which a cell, a tissue or its cell membrane fraction which contains a G protein-coupled receptor agonist or antagonist is first used to obtain candidate compounds (first screening) and, thereafter, whether candidate compounds actually inhibit binding of a human G protein-coupled  
10 receptor protein and a ligand is confirmed (secondary screening). When a cell, a tissue or a cell membrane fraction is used as it is, it was difficult to actually screen an agonist or an antagonist for a receptor protein of interest due to the presence of other receptors.

However, for example, the use of a human-derived protein of the present  
15 invention excludes the necessity of first screening and a compound which inhibits binding of a ligand and a G protein-coupled receptor protein can be effectively screened. Further, whether a screened compound is an agonist or an antagonist can be simply evaluated.

A method for screening of the present invention is explained more  
20 particularly.

First, as present protein or the like used in a method for screening of the present invention, any protein can be used as long as it contains the aforementioned present protein or the like, although a cell membrane fraction of an organ of a mammal containing present protein or the like is suitable. However, since a human-  
25 derived organ is obtained with very difficulty, a human-derived receptor protein or the

like which was expressed at a large amount using a recombinant is suitable for use in screening.

For preparing present protein or the like, the aforementioned method is used, although it is preferred that present DNA is expressed in a mammal cell or an insect cell. As a DNA fragment coding a protein portion of interest, a complementary DNA is used, being not limiting. For example, a gene fragment and a synthetic DNA may be used. In order to introduce a DNA fragment encoding present protein and express it effectively, the DNA fragment is preferably incorporated downstream of polyhedron promoter of nuclear polyhedrosis virus (NPV) belonging to genus Bacuvirus, a host of which is an insect, SV40-derived promoter, promoter of retrovirus, metallothionein promoter, human heat shock promoter, cytomegalovirus promoter and SR $\alpha$  promoter. Examination of an amount and quality of expressed receptor can be performed by the method known per se. For example, it can be performed according to a method described in the literature [Nambi, P. et al., The Journal of Biological Chemistry, J. Biol. Chem., vol.267, pp.19555-19559, 1992].

Therefore, in a method for screening of the present invention, as one containing present protein or the like, protein or the like which was purified by the method known per se may be used, or a cell containing the protein or the like may be used, or a membrane fraction of a cell containing the protein or the like may be used.

In a method for screening of the present invention, when a cell containing present protein or the like is used, the cell may be fixed with glutaraldehyde, formalin or the like. Fixation can be carried out according to the method known per se.

A cell containing present protein or the like refers to a host cell which expressed the protein or the like and, as a host cell, Escherichia coli, Bacillus subtilis, yeast, insect cell and animal cell are preferable.

A cell membrane fraction refers to a fraction containing a cell membrane obtained according to a method known per se at a large amount after a cell is ruptured. As a method for rupturing a cell, there are a method of squeezing a cell with a Potter-Elvehjem type homogenizer, rupture with Waring blender or Polytron (manufactured by Kinematica), rupture by ultrasound, rupture by ejecting a cell through a thin nozzle while pressing with a French press and the like. For fractionating a cell membrane, a fractionating method by centrifugation such as a fractionation centrifugation method and a density gradient centrifugation method are mainly used. For example, a ruptured cell solution is centrifuged at a low speed (500 rpm-3000 rpm) for a shorter period of time (usually, about 1 minute to 10 minutes), a supernatant is further centrifuged at a higher speed (15000 rpm-30000 rpm) usually for 30 minutes to 2 hours and the resulting precipitates are used as a membrane fraction. An expressed protein and a membrane component such as phospholipid and membrane protein derived from a cell are contained in the membrane fraction at a large amount.

An amount of a cell containing the protein or the protein in the membrane fraction is preferably  $10^3$ - $10^8$  molecules per 1 cell, suitably  $10^5$ - $10^7$  molecules. As an expression amount grows larger, the ligand binding activity (specific activity) is increased and, thereby, not only construction of high sensitive screening system becomes possible but also a large amount of samples can be measured at the same lot.

In order to perform the aforementioned methods (1)-(3) for screening a compound which alters property of a ligand binding with present protein or the like, for example, a suitable protein fraction and a labeled test compound are necessary.

As a protein fraction, a natural receptor protein fraction or a recombinant receptor fraction having the equivalent activity thereto are desirable. Here, equivalent activity denotes equivalent ligand binding activity, signal information

transmitting action and the like.

As a labeled test compound, a labeled ligand and a labeled ligand analogue compound are used. For example, a ligand labeled with [<sup>3</sup>H], [<sup>125</sup>I], [<sup>14</sup>C] or [<sup>35</sup>S] is used.

More particularly, in order to perform a method for screening a compound which alters property of a ligand binding with present protein or the like, an authentic protein is first prepared by suspending a cell containing present protein or the like or a membrane fraction of the cell in a buffer suitable for screening. As a buffer, any buffer such as phosphate buffer having pH 4-10 (desirably, pH 6-8) and Tris-HCl buffer may be used as long as it dose not inhibit binding of a ligand with a protein.

In addition, in order to reduce the non-specific binding, surfactants such as CHAPS, Tween-80<sup>TM</sup> (Kao-Atlas), digitonin and deoxycholate may be added to a buffer.

Further, in order to suppress degradation of a receptor or a ligand by protease, protease inhibiting agents such as PMSF, leupeptin, E-64 (manufactured by Peptide Laboratory) and pepstatin may be added thereto. A labeled ligand is added to 0.01 ml-10 ml of a solution of the receptor at a certain amount (5000 cpm-500000 cpm) and, at the same time, a test compound is present at  $10^{-4}$ M- $10^{-10}$ M. In order to know an amount of non-specific binding (NBS), a reaction tube to which a large excess unlabeled ligand is added is prepared. A reaction is performed at 0°C to 50°C, desirably at about 4°C to 37°C for about 20 minutes to 24 hours, desirably for about 30 minutes to 3 hours. After reaction, the reaction is filtered with a glass fiber filter or the like, washed with a suitable amount of a buffer, and the radioactivity remaining on a glass fiber filter is measured with a liquid scintillation counter or a  $\gamma$ -counter. A test compound having non-specific binding amount (B-NSB) (wherein a count (B0-NSB) obtained by subtracting non-specific binding amount (NSB) from a count (B0)

of no competing substance) can be selected as a candidate compound having the competition inhibiting ability.

In order to perform the aforementioned methods (4)-(5) for screening a compound which alters property of a ligand binding with present protein or the like,  
5 for example, the cell stimulating activity (for example, activity of promoting or inhibiting arachidonic acid release, acetylcholine release, intracellular Ca release, intracellular cAMP production, intracellular cGMP production, inositol phosphate production, intracellular potential variation, phosphorylation of an intracellular protein, c-fos activation, decrease in pH and the like) via a protein can be measured  
10 using the known method or a commercially available measuring kit.

More particularly, first, a cell containing present protein or the like is cultured on a multiwell-plate or the like. Upon implementation of screening medium is freshly prepared or buffer is changed to the proper one which does not denote toxicity for cell beforehand, a test compound and the like are added, incubated for a certain  
15 time, the cell is extracted or the supernatant is recovered to quantitate the resulting products according to respective methods. When the production of a substance (for example, arachidonic acid and the like) which is an index for the cell stimulating activity is difficult to be detected due to a degrading enzyme contained in the cell, an inhibiting agent for the degrading enzyme may be added to perform an assay. In  
20 addition, regarding the activity of cAMP production suppression, it can be detected as production inhibiting action for a cell for which fundamental producing amount has been increased with forskolin.

In order to perform screening by measuring the cell stimulating activity, a cell which expressed a suitable protein is necessary. As a cell which expressed present  
25 protein or the like, a cell strain containing natural type present protein or the like, and

a cell strain which expressed the aforementioned recombinant protein or the like are desirable.

As a test compound, for example, a peptide, a protein, a non-peptide compound, a synthetic compound, a fermented product, a cell extract solution, a plant extract solution, and an animal tissue extract solution are used, and these compounds may be a novel compound or the known compound.

A kit for screening a compound which alters property of a ligand binding with present protein or the like contains present protein or the like, a cell containing present protein or the like, or a membrane fraction of a cell containing present protein or the like.

As an example of a kit for screening of the present invention, there are the following ones:

1. A reagent for screening
- 15 (1) A buffer for measurement and a buffer for washing

0.05% bovine serum albumin (manufactured by Sigma) is added to Hanks' Balanced Salt Solution (manufactured by Gibco).

Filter-sterilized with a filter having a pore size of 0.45  $\mu\text{m}$ , which may be stored at 4°C or prepared upon use.

- 20 (2) An authentic G protein-coupled receptor

CHO cell in which a protein of the present invention is expressed is subcultured on a 12-well plate at  $5 \times 10^5$  cells/well, and cultured at 37°C, in 5% CO<sub>2</sub> and 95% air for two days.

- (3) A labeled ligand

25 A ligand labeled with commercially available [<sup>3</sup>H], [<sup>125</sup>I], [<sup>14</sup>C], [<sup>35</sup>S] or the

like. An aqueous solution is stored at 4°C or -20°C and diluted with a measuring buffer upon use.

(4) A standard solution of ligand

A ligand is dissolved to be 1 Mm with PBS which obtained 0.1 % bovine

5 serum albumin (manufactured by Sigma) and stored at -20°C

2. A measuring method

(1) CHO cell, expressing present protein, which was cultured on a 12-well

plate for tissue culturing is washed twice with a 1 ml of a measuring buffer, and a 490

10  $\mu$ l of a measuring buffer is added to each well.

(2) After 5  $\mu$ l of a  $10^{-3}$ - $10^{-10}$ M test compound is added, 5  $\mu$ l of a labeled

ligand is added, which is reacted at room temperature for 1 hour. In order to know

an amount of non-specific binding, 5  $\mu$ l of a  $10^{-3}$ M ligand is added instead of a test compound.

15 (3) The reaction solution is removed and washed with 1 ml of a washing

buffer three times. A labeled ligand bound to a cell is dissolved with 0.2N NaOH-

1% SDS, and mixed with 4 ml of liquid scintillator A (manufactured by

Wakojunyaku).

(4) The radioactivity is measured using a liquid scintillation counter

20 (manufactured by Beckmann) and Percent Maximum Binding (PMB) is obtained

according to the following equation [Mathematic 1]

[Mathematic 1]

$$\text{PMB} = [(B-\text{NSB})/(B_0-\text{NSB})] \times 100$$

25 PMB: Percent Maximum Binding

B: value when a specimen is added

NSB: Non-specific Binding (non-specific binding amount)

B0: Maximum binding amount

5        A compound obtained by using a method for screening or a kit for screening  
of the present invention or a salt thereof is a compound which alters property of a  
ligand binding with present protein or the like. More particularly, there are (a) a  
compound having the cell stimulating activity (for example, activity of promoting or  
suppressing arachidonic acid release, acetylcholin release, intracellular  $\text{Ca}^{2+}$  release,  
10      intracellular cAMP production, intracellular cGMP production, inositol phosphate  
production, cell membrane potential variation, phosphorylation of intracellular protein,  
activation of c-fos and decrease in pH) via a G protein-coupled receptor (so called  
agonist to present protein), (b) a compound having no such a cell stimulating activity  
(so called antagonist to present protein), (c) a compound which enhances binding  
15      force of a ligand with present G protein-coupled receptor, and (d) a compound which  
decreases binding force of a ligand with present G protein conjugated type protein.

As the compound, there are a peptide, a protein, a non-peptide compound, a  
synthetic compound and a fermented product, and these compounds may be a novel  
compound or the known compound.

20       Since an agonist to present protein or the like has the similar physiological  
activity to that of a ligand for present protein or the like, it is useful as a safe and low-  
toxic drug depending upon the ligand activity.

Since an antagonist to present protein or the like has the similar physiological  
activity to that of a ligand for present protein or the like, it is useful as a safe and low-  
25      toxic drug which inhibits the ligand activity.

A compound which enhances the binding force of a ligand binding with present protein is useful as a safe and low-toxic drug for enhancing the physiological activity harbored by a ligand for present protein or the like.

5 A compound which decreases the binding force of a ligand binding with present protein is useful as a safe and low-toxic drug for decreasing the physiological activity harbored by a ligand for present protein or the like.

A compound obtained by a screening method or a kit for screening of the present invention or a salt thereof can be used as the aforementioned pharmaceutical composition according to the conventional method. For example, it can be  
10 formulated into a tablet, a capsule, an elixir, a microcapsule, a sterile solution or a suspension solution as in the aforementioned drug containing present DNA.

Since pharmaceutical preparations thus obtained are safe and low toxic, they can be administered to, for example, human being or a mammal (for example, rat, rabbit, sheep, pig, cow, cat, dog, monkey).

15 A dose of the compound or a salt thereof is different depending upon an administration subject, a subject organ, symptom and an administration method and, in the case of oral administration, the dose is generally about 0.1 mg-100 mg, preferably about 1.0-50 mg, more preferably about 1.0-20 mg per day, in an adult (60 kg). When parentally administrated, one time dose is different depending upon an  
20 administration subject, a subject organ, symptom and an administration method and, in the form of injectable, it is usually advantageous to administer about 0.01-30 mg, preferably about 0.1-20 mg, more preferably about 0.1-10 mg by intravenous injection per day, in an adult (60 kg). In other animals, an amount calculated per 60 kg can be administrated.

(6) Quantitation of present protein , or a partial peptide thereof or a salt thereof

Since an antibody of the present invention can specifically recognize present protein or the like, it can be used for quantitation of present protein or the like in a specimen solution, in particular, quantitation by a sandwich immunodetection method.

- 5 That is, the present invention provides, for example, (i) a method for quantitating present protein or the like in a specimen solution, which comprises reacting competitively an antibody of the present invention with a specimen solution and a labeled protein or the like, and measuring a ratio of a labeled protein or the like bound to the antibody, (ii) a method for quantitating present protein or the like in a specimen
- 10 10 solution, which comprises reacting a specimen solution with an antibody of the present invention which is insolubilized on a carrier and a labeled antibody of the present invention simultaneously or successively, and measuring the activity of a labeling agent on an insolubilized carrier.

In the above (ii), it is preferred that one antibody is an antibody which  
15 recognizes a N-terminal of present protein or the like and an another antibody is an antibody which reacts with a C-terminal of present protein or the like.

By using a monoclonal antibody to present protein or the like (hereinafter, referred to as present monoclonal antibody in some cases), detection by tissue staining or the like can be conducted in addition to measurement of present protein or the like.  
20 For these purposes, an antibody molecule may be used or F(ab')2, Fab' or Fab fraction of an antibody may be used. A method for measurement using an antibody to present protein or the like is not particularly limited, but any measuring method may be used as long as it is a method for chemically or physically detecting an amount of an antibody, an antigen or an antibody-antigen complex corresponding to an amount of  
25 antigen in a specimen solution (for example, an amount of a protein), and calculating

this from a standard curve made using a standard solution containing the known amounts of an antigen. For example, nephrometry, competitive method, immunometric method and sandwich method are suitably used, although the use of a sandwich method described below is particularly preferable in a viewpoint of sensitivity and specificity.

As a labeling agent used in a measuring method using a labeling substance, for example, a radioisotope, an enzyme, a fluorescent substance and emitting substance are used. As a radioisotope, for example, [<sup>125</sup>I], [<sup>131</sup>I], [<sup>3</sup>H] and [<sup>14</sup>C] are used. As the above enzyme, enzymes which are safe and have the great specific activity are preferable. For example,  $\beta$ -galactosidase,  $\beta$ -glycosidase, alkaline phosphatase, peroxidase and malate dehydrogenase are used. As a fluorescent substance, for example, fluorescamine and fluorescein isothiocyanate are used. As an emitting substance, for example, luminol, luminol derivative, luciferin and lucigenin are used. Further, biotin-avidin system may be used for binding an antibody or an antigen and a labeling agent.

Upon insolubilization of an antigen or an antibody, physical adsorption may be used and, a method using chemical binding usually used for insolubilizing or immobilizing a protein or an enzyme may be used. As a carrier, for example, insoluble polysaccharides such as agarose, dextran and cellulose, synthetic resins such as polystyrene, polyacrylamide, silicone and the like, or glasses are used.

In a sandwich method, a specimen solution is reacted with an insolubilized monoclonal antibody of the present invention (primary reaction) and a labeled monoclonal antibody of the present invention is further reacted therewith (secondary reaction) and, thereafter, the activity of a labeling agent on an insolubilized carrier can be measured to quantitate an amount of present protein in a specimen solution. The

primary reaction and the secondary reaction may be performed in the reverse order, or simultaneously, or at a different time. A labeling agent and an insolubilizing method can be according to the aforementioned methods.

In addition, in an immunological detecting method by a sandwich method, an antibody used for a solid phase antibody or a labeling antibody is not necessarily one kind and a mixture of two or more kinds of antibodies may be used for improving the measuring sensitivity.

In a method for measuring present protein or the like, as monoclonal antibodies of the present invention used in the primary reaction and the secondary reaction, antibodies having a different site to which present protein or the like is bound are preferably used. That is, regarding antibodies used in the primary reaction and the secondary reaction, an antibody used in the primary reaction is preferably an antibody which recognizes a site other than a C-terminal, for example, a N-terminal when an antibody used in the secondary reaction recognizes a C-terminal of present protein.

A monoclonal antibody of the present invention can be used a measuring system other than a sandwich method, for example, competitive method, immunometric method or nephrometry. In a competitive method, after an antigen in a specimen solution and a labeled antigen are reacted competitively with an antibody, an unreacted labeled antigen (F) and a labeled antigen (B) bound to an antibody are separated (B/F separation), and a labeled amount of either of B or F is measured to quantitate an amount of an antigen in a specimen solution. In the present reaction, there are used a solution method in which a soluble antibody is used as an antibody, B/F separation is performed using polyethylene glycol and the second antibody to the aforementioned antibody, and a solid phase method in which a soluble antibody is

used as the first antibody and a solid phased antibody is used as the second antibody.

In an immunometric method, after an antigen in a specimen solution and a solid phased antigen are reacted competitively with a constant amount of labeled antibody, the solid phase and the solution phase are separated, or after an antigen in a specimen solution and an excessive amount of labeled antibody are reacted and, then, a solid phased antigen is added to bind unreacted labeled antibody to a solid phase, the solid phase and the solution phase are separated. Next, a labeled amount of either phase is measured to quantitate an amount of an antigen in a specimen solution.

In addition, in nephrometry, an amount of insoluble presipitates resulted from an antigen-antibody reaction in a gel or a solution is measured. Also when an amount of an antigen in a specimen solution is small and a small amount of presipitates are obtained, laser nephrometry using laser scattering is used.

Upon application of individual immunological measuring methods to the present measuring method, setting of special conditions and operations are not required. A system for measuring present protein or a salt thereof may be constructed by addition of technical consideration by those skilled in the art to the conventional conditions and operation procedures in respective methods. For details of these general technical means, a reference can be made to reviews and books [for example, see "Radioimmunoassay" edited by Hiroshi Irie (Kodansha, published in 1974), "Radioimmunoassay, Second Series" edited by Hiroshi Irie (Kodansha, published in 1979), "Enzyme Immunoassay" edited by Eiji Ishikawa et al. (Igakushoin, published in 1978), "Enzyme Immunoassay" edited by Eiji Ishikawa et al. (2nd edition) (Igakushoin, published in 1982), "Enzyme Immunoassay" edited by Eiji Ishikawa et al. (3rd edition) (Igakushoin, published in 1987), "Methods in ENZYMOLOGY" Vol. 70 (Immunochemical Techniques (Part A)), Ibid. Vol. 73

(Immunochemical Techniques (Part B)), Ibid. Vol. 74 (Immunochemical Techniques (Part C)), Ibid. Vol. 84 (Immunochemical Techniques (Part D: Selected Immunoassay)), Ibid. Vol. 92 (Immunochemical Techniques (Part E: Monoclonal Antibodies and Generals Immunoassay Methods)), Ibid. Vol. 121 (Immunochemical Techniques (Part I: Hybridoma Technology and Monoclonal Antibodies)) (All published by Academic Press).

As described above, by using an antibody of the present invention, present protein or a salt thereof can be quantitated with the high sensitivity. Further, various diseases can be diagnosed by quantitating present protein or a salt thereof using an antibody of the present invention.

In addition, an antibody of the present invention can be used to detect present protein or the like present in a specimen such as a body liquid or a tissue. In addition, it can be used for manufacturing an antibody column used for purifying present protein or the like, detecting present protein or the like in each fraction upon purification, and analyzing the behavior of present protein or the like in a specimen cell.

(7) Preparation of non-human animal having a DNA encoding a G protein conjugated type protein of the present invention

A transgenic non-human animal expressing present protein or the like can be made using a DNA of the present invention. As a non-human animal, there is a mammal (for example, rat, mouse, rabbit, sheep, pig, cow, cat, dog, monkey and the like) (hereinafter, abbreviated as animal), although mouse and rabbit are particularly suitable.

Upon transference of a DNA of the present invention to a subject animal, it is

generally advantageous to use as a gene construct bound downstream of promoter which can express the DNA in an animal. For example, when a DNA of the present invention derived from a rabbit is transferred, a DNA-transferred animal which produces present protein or the like at a large amount can be produced by introducing 5 a gene construct in which a DNA of the present invention, derived from an animal, having high homology with the above DNA is bound downstream of various promoters which can express a DNA of the present invention in an animal cell, into rabbit fertilized ovum by microinjection. As this promoter, for example, ubiquitous expression promoters such as virus-derived promoter, metallothionein promoter and 10 the like may be also used, although NGF gene promoter and enolase gene promoter which are specifically expressed in brain are preferably used.

Transference of a DNA of the present invention at fertilized ovum cell stage is maintained such that the DNA is present in all of an embryo cell and a somatic cell of a subject animal. The presence of present protein or the like in an embryo cell in a 15 produced animal after DNA transference means that all progenies of produced animal have present protein or the like in their all embryo cells and somatic cells. A progeny of such the animal which inherited a gene has present protein or the like in all its embryo cells and somatic cells.

A DNA transferred animal of the present invention can be passage-bred as an 20 animal harboring the DNA under the normal breeding circumstances by assuring that a gene is stably retained by mating. Further, a homozygote animal having an introduced gene in both of homologous chromosomes can be obtained by mating male and female animals retaining a DNA of interest and breeding passage may be performed such that all progenies have the DNA, by mating the male and female.

25 Since an animal, in which a DNA of the present invention is introduced,

expresses highly present protein or the like, it is useful as an animal for screening an agonist or an antagonist for present protein or the like.

A DNA-transferred animal of the present invention may be used as a cell source for tissue culturing. For example, present protein or the like can be analyzed 5 by analyzing directly a DNA or a RNA in a tissue of a DNA-transferred mouse of the present invention, or by analyzing a tissue in which a protein of the present invention expressed by a gene is present. A cell of a tissue having present protein or the like is cultured by standard tissue culturing technique, which can be used to study the function of a cell from a tissue which is generally difficult to culture, such as derived 10 from brain or peripheral tissue. In addition, by using the cell, for example, a drug which enhances the function of various tissues can be selected. In addition, when there is a high expressing cell strain, present protein or the like can be also isolated and purified therefrom.

When a nucleotide, an amino acid and the like are expressed as an 15 abbreviation in the present invention and drawings, the abbreviation is based on abbreviation by IUPAC-IUB Commission on Biochemical Nomenclature or the conventional abbreviation in the art. Examples thereof are shown below. When there are optical isomers regarding an amino acid, L amino acid is shown unless indicated.

20 DNA: deoxyribonucleic acid

cDNA: complementary deoxyribonucleic acid

A: adenine

T: thymine

G: guanine

25 C: cytosine

- RNA: ribonucleic acid
- mRNA: messenger ribonucleic acid
- dATP: deoxyadenosine triphosphate
- dTTP: deoxythymidine triphosphate
- 5 dGTP: deoxyguanosine triphosphate
- dCTP: deoxycytidine triphosphate
- ATP: adenosine triphosphate
- EDTA: ethylenediamine tetraacetic acid
- SDS: sodium dodecylsulfate
- 10 Gly: glycine
- Ala: alanine
- Val: valine
- Leu: leucine
- Ile: isoleucine
- 15 Ser: serine
- Thr: threonine
- Cys: cysteine
- Met: methionine
- Glu: glutamic acid
- 20 Asp: aspartic acid
- Lys: lysine
- Arg: arginine
- His: histidine
- Phe: phenylalanine
- 25 Tyr: tyrosine

Trp: tryptophan

Pro: proline

Asn: asparagine

Gln: glutamine

5 pGlu: pyroglutamic acid

Me: methyl group

Et: ethyl group

Bu: butyl group

Ph: phenyl group

10 TC: thiazolidine-4(R)-carboxamide group

In addition, substituents, protecting groups and reagents which are frequently used are expressed by the following symbols:

Tos: p-toluenesulfonyl

CHO: formyl

15 Bzl: benzyl

Cl<sub>2</sub>Bzl: 2,6-dichlorobenzyl

Bom: benzyloxymethyl

Z: benzyloxycarbonyl

Cl-Z: 2-chlorobenzyloxycarbonyl

20 Br-Z: 2-bromobenzyloxycarbonyl

Boc: t-butoxycarbonyl

DNP: dinitrophenol

Trt: trityl

Bum: t-butoxymethyl

25 Fmoc: N-9-fluorenylmethoxycarbonyl

HOBt: 1-hydroxybenztriazole

HOOBt: 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine

HONB: 1-hydroxy-5-norbornene-2,3-dicarboximide

DCC: N,N'-dicyclohexylcarbodiimide

5 Sequence numbers of SEQ ID Tables in the present specification show the following sequences.

[SEQ ID No:1]

An amino acid sequence of a human brain-derived protein of the present invention is shown.

10 [SEQ ID No:2]

A nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention having an amino acid represented by SEQ ID No:1.

[SEQ ID No:3]

An amino acid sequence of a human brain-derived protein of the present invention is shown.

[SEQ ID No:4]

A nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention having an amino acid represented by SEQ ID No:3.

[SEQ ID No:5]

20 An amino acid sequence of a human brain-derived protein of the present invention is shown.

[SEQ ID No:6]

A nucleotide sequence of a DNA encoding a human brain-derived protein of the present invention having an amino acid represented by SEQ ID No:5.

25 Transformant Escherichia coli JM109/pHK05006 obtained in Example 1

below has been deposited at Trade and Commerce Ministry Industrial Technology Institute Life Technology Industrial Technology Laboratory (NIBH) under accession number of FERM BP-6433 since July 21, 1998 and at Foundation Fermentation Laboratory under accession number of IFO 16189 since July 8, 1998.

5        Transformant Escherichia coli JM109/pHK05490 obtained in Example 2 below has been deposited at Trade and Commerce Ministry Industrial Technology Institute Life Technology Industrial Technology Laboratory (NIBH) under accession number of FERM BP-6456 since August 7, 1998.

10      Transformant Escherichia coli JM109/pHH02631 obtained in Example 3 below has been deposited at Trade and Commerce Ministry Industrial Technology Institute Life Technology Industrial Technology Laboratory (NIBH) under accession number of FERM BP-6540 since October 6, 1998.

#### Examples

15        The following Examples illustrate the present invention but do not limit the scope thereof. Gene manipulations using Escherichia coli were according to a method described in Molecular cloning.

#### Example 1

20        Search of a human brain-derived G protein-coupled receptor protein and determination of a nucleotide sequence (1)

Homology search was made to amino acid sequences encoded by cDNAs obtained by a method described in DNA RESEARCH, vol.4, pp.53-59 (1997) or a similar method using sequences of the known receptors; in particular, sequences of 25        human ORF receptors as a template, and an amino acid sequence represented by SEQ

ID No:1 (amino acid sequence in Fig. 1 and Fig. 2) showed homology.

Then, cDNA corresponding to amino acid sequence in SEQ ID No:1 (amino acid sequence in Fig. 1 and Fig. 2) was sequence-analyzed and SEQ ID No:2 (nucleotide sequence in Fig. 1 and Fig. 2) was obtained. Hydrophobicity plot shown 5 in Fig. 3 was obtained and it was found that seven times transmembrane type (G protein conjugated type) receptor is encoded.

Further, plasmid pHK05006 harboring a DNA represented by SEQ ID No:2 was introduced into E. coli JM109 to obtain E. coli JM109/pHK05006.

10 Example 2

Search of a human brain-derived G protein-coupled receptor protein and determination of a nucleotide sequence (2)

Homology search was made to amino acid sequences encoded by cDNAs [SEQ ID No:4 (DNA sequence in Fig.7-Fig. 15)] obtained by a method described in 15 DNA RESEARCH, vol.4, pp.53-59 (1997) or a similar method using sequences of the known receptors; in particular, sequences of human ORF receptors as a template, and an amino acid sequence represented by SEQ ID No:3 (amino acid sequence represented by HKO05490 in Fig. 4 and Fig. 5; amino acid sequence in Fig. 7-Fig.15) showed homology.

20 Then, hydrophobicity plot was analyzed and this is shown in Fig. 6, and it was found that seven times transmembrane type (G protein conjugated type) receptor is encoded. In addition, it was confirmed that a protein represented by an amino acid sequence represented by SEQ ID No:3 shows high homology with a protein represented by an amino acid sequence represented by SEQ ID No:1 described in

25 Example 1 (Fig.4).

Further, plasmid pHK05490 harboring a DNA represented by SEQ ID No:4 was introduced into E. coli JM109 to obtain E. coli JM109/pHK05490.

### Example 3

5       Search of a human brain-derived G protein-coupled receptor protein and determination of a nucleotide sequence (1)

Homology search was made to amino acid sequences encoded by cDNAs obtained by a method described in DNA RESEARCH, vol.4, pp.53-59 (1997) or a similar method using sequences of the known receptors; in particular, sequences of 10 human ORF receptors as a template, and an amino acid sequence represented by SEQ ID No:5 showed homology.

Then, cDNA corresponding to amino acid sequence in SEQ ID No:5 was sequence-analyzed and SEQ ID No:6 was obtained. Hydrophobicity plot shown in Fig. 25 was obtained and it was found that seven times transmembrane type (G protein 15 conjugated type) receptor is encoded.

Further, plasmid pHH02631 harboring a DNA represented by SEQ ID No:6 was introduced into E. coli JM109 to obtain E. coli JM109/pHH02631.

### Industrial Applicability

20       A protein of the present invention, a partial peptide thereof or a salt thereof, and a DNA encoding it can be used in or as (1) determination of a ligand (agonist), (2) obtaining of an antibody and antiserum, (3) construction of the expression system for a recombinant protein, (4) development of the receptor binding assay using the same expression system and screening of drug candidates, (5) implementation of drug 25 design based on comparison with a ligand receptor having the structural similarity, (6)

reagents for preparing a probe or a PCR primer in gene therapy, (7) production of a transgenic animal, or (8) a drug for gene prophylaxis or therapy.

What is claimed is:

1. A protein comprises the same or substantially the same amino acid sequence represented by SEQ ID No:1, SEQ ID No:3 or SEQ ID No:5, or a salt thereof.
- 5 2. A partial peptide of the protein according to claim 1, or a salt thereof.
3. A DNA comprises a DNA having a nucleotide sequence encoding the protein according to claim 1.
4. The DNA according to claim 3, which comprising a nucleotide sequence represented by SEQ ID No:2, SEQ ID No:4 or SEQ ID No:6.
- 10 5. A recombinant vector comprising the DNA according to claim 3.
6. A transformant transformed with the recombinant vector according to claim 5.
7. A process for producing the protein or a salt thereof according to claim 1, which comprises culturing the transformant according to claim 6, and producing and
- 15 15. accumulating the protein according to claim 1.
8. An antibody to the protein according to claim 1, the partial peptide according to claim 2 or a salt thereof.
9. A method for determining a ligand for the protein according to claim 1 or a salt thereof, which comprises using the protein according to claim 1, the partial peptide according to claim 2 or a salt thereof.
- 20 10. A method for screening a compound or a salt thereof which alters property of a ligand binding with the protein according to claim 1 or a salt thereof, which comprises using the protein according to claim 1, the partial peptide according to claim 2 or a salt thereof.
- 25 11. A kit for screening a compound or a salt thereof which alters property of

a ligand binding with the protein according to claim 1 or a salt thereof, which comprises the protein according to claim 1, the partial peptide according to claim 2 or a salt thereof.

12. A compound or a salt thereof which alters property of a ligand binding

5 with the protein according to claim 1 or a salt thereof, which is obtainable by the method for screening according to claim 10 or the kit for screening according to claim 11.

13. A pharmaceutical which comprises a compound or a salt thereof which

10 alters property of a ligand binding with the protein according to claim 1 or a salt thereof, which is obtainable by the method for screening according to claim 10 or the kit for screening according to claim 11.

14. A DNA which hybridizes with the DNA according to claim 3 under

stringent conditions.

15. A nucleotide containing a nucleotide sequence encoding the protein

15 according to claim 1.

16. A nucleotide containing a part of a nucleotide sequence which is

complementary to a nucleotide sequence encoding the protein according to claim 1.

SEQUENCE LISTING

<110> Takeda Chemical Industries, Ltd.

<120> Novel G Protein Coupled Receptor Protein and Its Use

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5 <150> JP 10-207579

<151> 1998-07-23

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<151> 1998-08-07

<150> JP 10-284328

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1

5

10

15

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20

20

25

30

Asn Leu Thr Pro Gly Gly Lys Asp Ser Ala Ala Arg Ser Leu Asn Lys  
                  35                 40                 45  
 Ala Met Val Glu Thr Val Asn Asn Leu Leu Gln Pro Gln Ala Leu Asn  
                  50                 55                 60  
 5 Ala Trp Arg Asp Leu Thr Thr Ser Asp Gln Leu Arg Ala Ala Thr Met  
        65                 70                 75                 80  
 Leu Leu His Thr Val Glu Glu Ser Ala Phe Val Leu Ala Asp Asn Leu  
                  85                 90                 95  
 Leu Lys Thr Asp Ile Val Arg Glu Asn Thr Asp Asn Ile Lys Leu Glu  
 10             100             105             110  
 Val Ala Arg Leu Ser Thr Glu Gly Asn Leu Glu Asp Leu Lys Phe Pro  
        115                 120                 125  
 Glu Asn Met Gly His Gly Ser Thr Ile Gln Leu Ser Ala Asn Thr Leu  
        130                 135                 140  
 15 Lys Gln Asn Gly Arg Asn Gly Glu Ile Arg Val Ala Phe Val Leu Tyr  
        145                 150                 155                 160  
 Asn Asn Leu Gly Pro Tyr Leu Ser Thr Glu Asn Ala Ser Met Lys Leu  
        165                 170                 175  
 Gly Thr Glu Ala Leu Ser Thr Asn His Ser Val Ile Val Asn Ser Pro  
 20             180             185             190  
 Val Ile Thr Ala Ala Ile Asn Lys Glu Phe Ser Asn Lys Val Tyr Leu  
        195                 200                 205  
 Ala Asp Pro Val Val Phe Thr Val Lys His Ile Lys Gln Ser Glu Glu  
        210                 215                 220  
 25 Asn Phe Asn Pro Asn Cys Ser Phe Trp Ser Tyr Ser Lys Arg Thr Met

225                    230                    235                    240  
Thr Gly Tyr Trp Ser Thr Gln Gly Cys Arg Leu Leu Thr Thr Asn Lys  
245                    250                    255  
Thr His Thr Thr Cys Ser Cys Asn His Leu Thr Asn Phe Ala Val Leu  
5                        260                    265                    270  
Met Ala His Val Glu Val Lys His Ser Asp Ala Val His Asp Leu Leu  
275                    280                    285  
Leu Asp Val Ile Thr Trp Val Gly Ile Leu Leu Ser Leu Val Cys Leu  
290                    295                    300  
10    Leu Ile Cys Ile Phe Thr Phe Cys Phe Phe Arg Gly Leu Gln Ser Asp  
305                    310                    315                    320  
Arg Asn Thr Ile His Lys Asn Leu Cys Ile Ser Leu Phe Val Ala Glu  
325                    330                    335  
Leu Leu Phe Leu Ile Gly Ile Asn Arg Thr Asp Gln Pro Ile Ala Cys  
15                       340                    345                    350  
Ala Val Phe Ala Ala Leu Leu His Phe Phe Phe Leu Ala Ala Phe Thr  
355                    360                    365  
Trp Met Phe Leu Glu Gly Val Gln Leu Tyr Ile Met Leu Val Glu Val  
370                    375                    380  
20    Phe Glu Ser Glu His Ser Arg Arg Lys Tyr Phe Tyr Leu Val Gly Tyr  
385                    390                    395                    400  
Gly Met Pro Ala Leu Ile Val Ala Val Ser Ala Ala Val Asp Tyr Arg  
405                    410                    415  
Ser Tyr Gly Thr Asp Lys Val Cys Trp Leu Arg Leu Asp Thr Tyr Phe  
25                       420                    425                    430

Ile Trp Ser Phe Ile Gly Pro Ala Thr Leu Ile Ile Met Leu Asn Val  
 435 440 445  
 Ile Phe Leu Gly Ile Ala Leu Tyr Lys Met Phe His His Thr Ala Ile  
 450 455 460  
 5 Leu Lys Pro Glu Ser Gly Cys Leu Asp Asn Ile Lys Ser Trp Val Ile  
 465 470 475 480  
 Gly Ala Ile Ala Leu Leu Cys Leu Leu Gly Leu Thr Trp Ala Phe Gly  
 485 490 495  
 Leu Met Tyr Ile Asn Glu Ser Thr Val Ile Met Ala Tyr Leu Phe Thr  
 10 500 505 510  
 Ile Phe Asn Ser Leu Gln Gly Met Phe Ile Phe Ile Phe His Cys Val  
 515 520 525  
 Leu Gln Lys Lys Val Arg Lys Glu Tyr Gly Lys Cys Leu Arg Thr His  
 530 535 540  
 15 Cys Cys Ser Gly Lys Ser Thr Glu Ser Ser Ile Gly Ser Gly Lys Thr  
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Lys Asn Leu Cys Ile Asn Leu Phe Leu Ala Glu Leu Leu Phe Leu Val  
900 905 910

15 Gly Ile Asp Lys Thr Gln Tyr Glu Ile Ala Cys Pro Ile Phe Ala Gly  
915 920 925

Leu Leu His Tyr Phe Phe Leu Ala Ala Phe Ser Trp Leu Cys Leu Glu  
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Gly Val His Leu Tyr Leu Leu Leu Val Glu Val Phe Glu Ser Glu Tyr  
20 945 950 955 960

Ser Arg Thr Lys Tyr Tyr Tyr Leu Gly Gly Tyr Cys Phe Pro Ala Leu  
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Val Val Gly Ile Ala Ala Ala Ile Asp Tyr Arg Ser Tyr Gly Thr Glu  
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25 Lys Ala Cys Trp Leu Arg Val Asp Asn Tyr Phe Ile Trp Ser Phe Ile

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	1010	1015	1020
Thr Leu His Lys Met Ile Arg Ser Ser Ser Val Leu Lys Pro Asp Ser			
5	1025	1030	1035
Ser Arg Leu Asp Asn Ile Lys Ser Trp Ala Leu Gly Ala Ile Ala Leu			
	1045	1050	1055
Leu Phe Leu Leu Gly Leu Thr Trp Ala Phe Gly Leu Leu Phe Ile Asn			
	1060	1065	1070
10	Lys Glu Ser Val Val Met Ala Tyr Leu Phe Thr Thr Phe Asn Ala Phe		
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Gln Gly Val Phe Ile Phe Val Phe His Cys Ala Leu Gln Lys Lys Val			
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His Lys Glu Tyr Ser Lys Cys Leu Arg His Ser Tyr Cys Cys Ile Arg			
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Ser Asn Thr Arg Tyr Tyr Gly Thr Gln Ser Arg Ile Arg Arg Met			
	1140	1145	1150
20	Trp Asn Asp Thr Val Arg Lys Gln Thr Glu Ser Ser Phe Met Ala Gly		
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Asp Ile Asn Ser Thr Pro Thr Leu Asn Arg Gly Thr Met Gly Asn His			
	1170	1175	1180
Leu Leu Thr Asn Pro Val Leu Gln Pro Arg Gly Gly Thr Ser Pro Tyr			
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1285 1290 1295

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1300 1305 1310

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1315 1320 1325

Asp Arg Ala Glu Ile Glu Leu Leu Tyr Lys Ala Leu Glu Glu Pro Leu  
1330 1335 1340

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20 1345 1350 1355 1360

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Ser Pro Pro Gly Arg Asp Ser Leu Tyr Ala Ser Gly Ala Asn Leu Arg  
1380 1385 1390

25 Asp Ser Pro Ser Tyr Pro Asp Ser Ser Pro Glu Gly Pro Ser Glu Ala

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 1410                    1415                    1420  
 Thr Ser Arg Pro Pro Ala Leu Val Ala Arg Asn Pro Leu Gln Gly Tyr  
 5    1425                    1430                    1435                    1440  
 Tyr Gln Val Arg Arg Pro Ser His Glu Gly Tyr Leu Ala Ala Pro Gly  
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 CAGTGCCTGG TGGTCGCCGG CTCGGATGCC TTTCTGACC CCTGTCTGG GACCTACAAG 360  
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097465 • CTCGCA  
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CGCTGGGGCG GAAAGACCGA CATTGACCTG GCGGTGGACG AGAACGGCT GTGGGTAC 840  
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15

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Figure 1

1	GCTAACAGACAAGAAATCACTTGAATGCTGGGACATCACCTACTCTGTCCGGGCCATG	60
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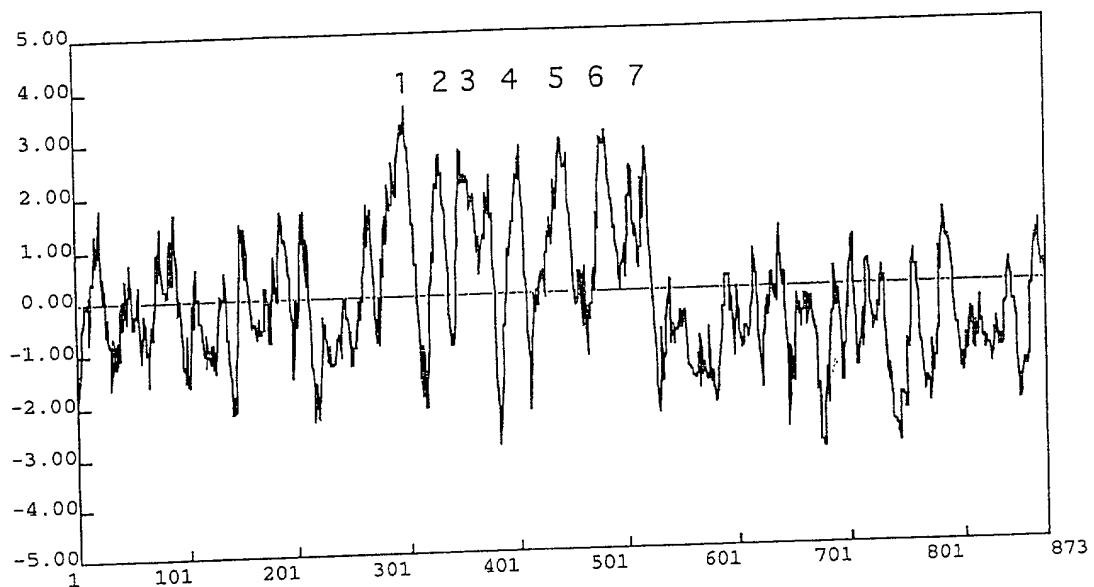
Figure 2

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2041	TCCAGTGAACACAAACAGGAATCTGATGAAACAAGCTGGTGAATAACCTGGCAGTGGAAAGG 681 SerSerGluGlnAsnAspLysLeuMetAsnLysLeuValAsnLysLeuGlySerGlyArg	2100 700
2101	GAAGATGATGCCATTGTCCTGGATGATGCCACCTCGTTAACACAGGAGAGTTGGGC 701 GluAspAspAlaIleValLeuAspAspAlaThrSerPheAsnHisGluGluSerLeuGly	2160 720
2161	CTGGAACTCATTGAGGAATCTGATGCTCTTGTGCCCCAAGAGTATACTCCACC 721 LeuGluLeuIleHisGluGluSerAspAlaProLeuLeuProProArgValTyrSerThr	2220 740
2221	GAGAACCCCCAACACCATTATACCAGAAGGCCATCCCCAAGACCACAGTGAGAGC 741 GluAsnHisGlnProHisHisTyrThrArgArgArgIleProGlnAspHisSerGluSer	2280 760
2281	TTTTCCCTTGCTAACCAACAGGACACAGAACAGAAGTCTCCAGTCACCCCATAAGAGACTCT 761 PhePheProLeuLeuThrAsnGluHisThrCysAspLeuGlnSerProHisArgAspSer	2340 780
2341	CTCTATACCAGCATGCCGACACTGGCTGGTGGCCGUCACAGAGAGTGTACCCACCAGC 781 LeuTyrThrSerMetProThrLeuAlaGlyValAlaAlaThrGluSerValThrThrSer	2400 800
2401	ACCCAGACCGAACCCCCACCGCCAAATGTGGTGTGCCGAAGATGTTACTACAAAAGC 801 ThrGlnThrGluProProAlaLysCysGlyAspAlaGluAspValTyrTyrLysSer	2460 820
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2521	CGCGGCAGCACTGATGGATTATAGTCCTCCAAACAAAGATGGGACCCCTCCGAGGG 841 ArgGlySerSerAspGlyPheIleValProProAsnLysAspGlyThrProProGluGly	2580 860
2581	AGTCAAAGGACCGGCTCATTGGTCACTAGTCTATAGAAGATGACACAGAAATTGGAA 861 SerSerLysGlyProAlaHisLeuValThrSerLeu***	2640 873
2641	CCAACAAAATGCTAACACCTGTTGACTGTTCTGAGTTGATATAAGCAGTGGATAATA 873	2700 873
2701	GTGTGTAATCCTAAATCTTATGCTGCTCTAAAGACAAACAAACTCTCAGACTTT 873	2760 873
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2941	ACATCTCTGATGCTGTGTTACTAAAATTACAAGGACCTGCTTTAAAAGGCCAGAACAA 873	3000 873

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Figure 3



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Figure 4

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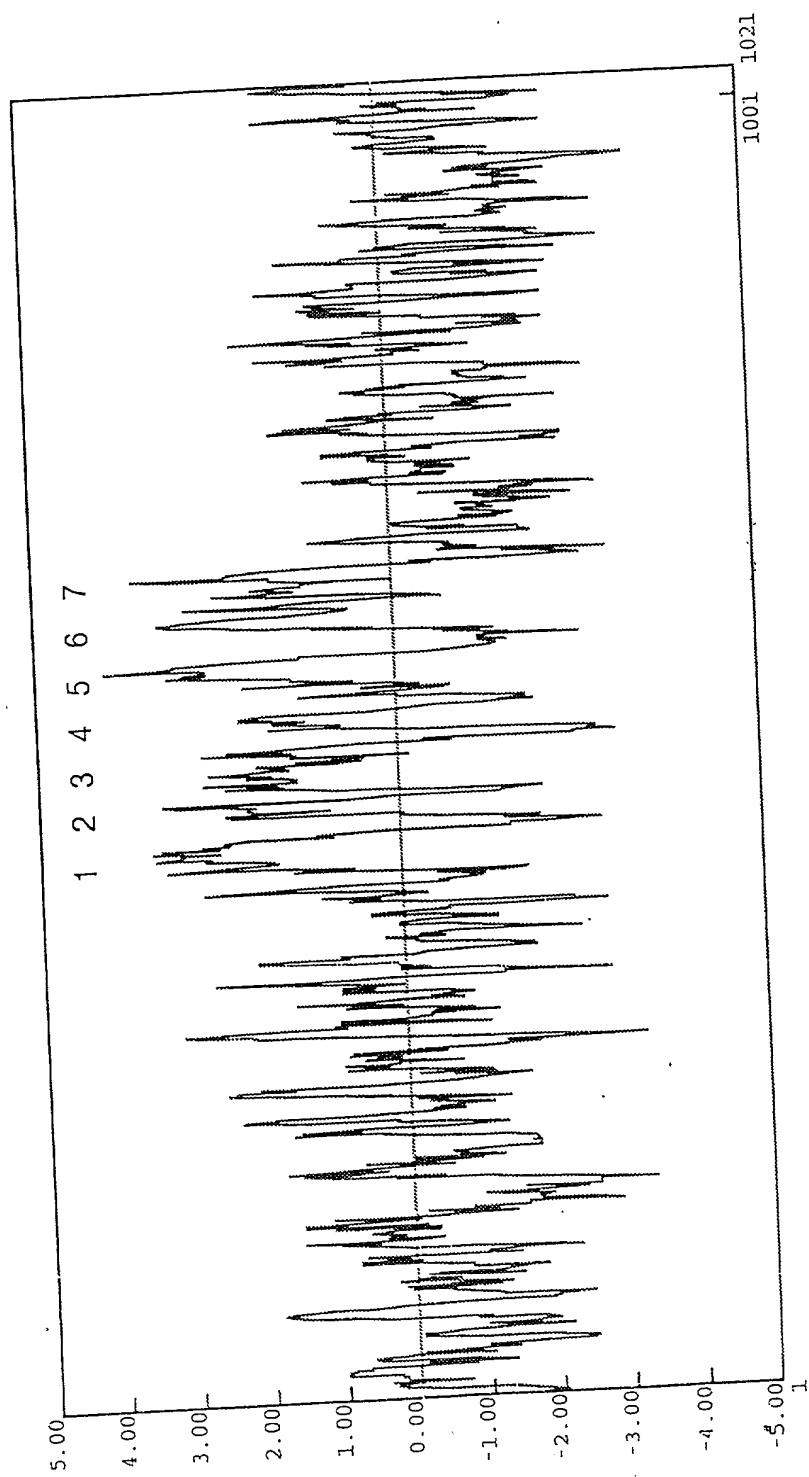
Figure 5

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479	V I G A I A L L C L L G L T W A F G I M Y I N E S T Y I M A Y L F T I F N S L O G M F I F H C V	HK05006
599	V L G A F A L L C L L G L T W S F G C L L F I N E E T I V M A Y L F T I E N A F Q G V F I F H C A	HK05490
529	L Q K K V R K E Y G K C L R - T H C C S G K S T E S S I G S G K T S G S R T P G R Y S T G S Q S R I	HK05006
649	L Q K K V R K E Y G K C F R H S Y C C G G L P T E S P H S S V K A S T T R T S A R Y S S G T Q S R I	HK05490
578	R R M W N D T V R K O S E S S F I T G D I N S S A S L N R E G L - - - - -	HK05006
699	R R M W N D T V R K O S E S S F I S G D I N S T S T L N - Q G M T G N Y L L T N E L L R P H G T N N	HK05490
610	- - - - -	HK05006
748	P Y N T L L A E T V C N A P S A P V F N S P G H S L N N A R D T S A M D T L P L N G N F N N S Y S	HK05490
634	I A S G E Y L S N C V Q L I D R G Y N H N E T A L E K K I L K E L T S N Y I P S Y L N N H E R S S E	HK05006
798	I H K G D Y - N D S V Q V V D C G L S L N D T A F E K M I I S E L - - - - -	HK05490
684	C N R N L K N K L Y N N - L G S G R E D D A I V L D A T S F N H E E S S L G E L L I H E S D A P	HK05006
839	K T H N L E L T I P V K P V I G G S S S E D D A I V A D A S S I M H E S D N P G L E L R H K E L E A P	HK05490
732	L L P P R V Y S T E N H Q P H H Y T R R I P Q D H S E S F P P L T N E H T E D L Q S P H R D S L	HK05006
889	L I P Q R T H S L L - Y Q P Q - - - K V K S E G T D S Y V S Q L T A E D H L O S P N R D S L	HK05490
782	Y T S M P T L A G V A A T E S V T S T Q T E P P P A K C G D A E D V Y Y K S M P N L G S R N H V F	HK05006
934	Y T S M P N L R D S P Y P E S - S P D M E E D L S P S R R S E N E D T Y Y K S M P N L G A - - - G H	HK05490
832	Q L H T Y Y Q L G R G S S D G F I V P P N K D G T P P E G S S K - G P A H L V T S L	HK05006
980	Q L Q M C Y Q I S R G N S D G Y I I P I N K E G C I P E G D V R E G O M Q L V T S L	HK05490

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Figure 6



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Figure 7

1 GAGGAAAGGCAAAACCACCTCAGCAGTTCTACAACCAAATTCCACCTATA 60  
1 Glu Gly Ser Lys Gly Thr Lys Pro Pro Ala Val Ser Thr Thr Lys Ile Pro Pro Ile 20

61 ACAATATTTCCTGCCAGAGAGATTCTGTGAGCATTAGACTCCAGGGATAAAAG 120  
21 Thr Asn Ile Phe Pro Leu Pro Glu Arg Phe Cys Glu Al Leu Asp Ser Lys Gly Ile Lys 40

121 TGGCTCTAGACACAAAGGGAAATGATCGTGAACGACCATGCCCTAAGGGAAACAGGAA 180  
41 Tri Pro Gln Thr Glu Lys Arg Gly Met Val Glu Arg Pro Cys Pro Lys Gly Thr Arg Gly 60

181 ACTGGCTCTATCTCTGCATGATTCCACTTGAAACATGGAAACCTTAAGGGCCCCGATCTT 240  
61 Thr Ala Ser Tyr Ile Cys Met Ile Ser Thr Gly Thr Trp Asn Pro Asp Leu 80

241 AGCAACTCTACCTCACACTGGTGAAATCAGCTGGCTCAGAACGATCAGAACGGGAGAAAT 300  
81 Ser Asn Cys Thr Ser His Trp Val Asn Gln Leu Ala Gln Lys Ile Arg Ser Gly Glu Asn 100

301 GCTGGCTAGTCCTCCANTGGAAACTGGCTAACATACCAAGGCCAGTGTGCTGGGGAT 360  
101 Ala Ala Ser Leu Ala Asn Gln Leu Ala Lys His Thr Lys Gly Pro Val Phe Ala Gly Asp 120

361 GTAAAGCTCTAGCTAGATGATGAGCAGCTGGGACATTCCTGATGCAACAGCTGAG 420  
121 Val Ser Ser Val Arg Leu Met Glu Gln Leu Val Asp Ile Leu Asp Alanine Gln Leu Gln 140

421 GAACTGAAACCTAGTGA AAAAAGATTCAAGCTGGACGGAGTTATAACAAGCTCCAAAACGA 480  
141 Glu Leu Lys Pro Ser Glu Lys Asp Ser Ala Gly Arg Ser Tyr Asn Lys Leu Gln Lys Arg 160

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Figure 8

481 GAGAGACATCAGGCTTACCTTAACGGCAATTGTGACAGTGACACCTTCAGA 540  
161 GluLysThrCysArgAlaTyrLeuLysAlaIleValAspThrValAspSerLeuLeuArg 180  
  
541 CCTCGAGCTTGAAATCATCGAACATATGAATTCATTCCTGAAACAATACTTGCAACA 600  
181 ProGluAlaLeuGluSerTerpLysHsMetAsnSerSerGluGlnAlaHisThrAlaThr 200  
  
601 ATGGTACTCGATACTTGAAAGAGGAGCTTGTGACAATCTTTAGAACCCA 660  
201 MetLeuLysAspThrLeuGluGlyLysAlaPheValLeuAlaAspAsnLeuGluPro 220  
  
661 ACAAGGGTCTCAATCCCCACAGAAATTATTCGCAACTTCGCTTCAGTACAGAA 720  
221 ThrArgValSerMetProThrGluAsnIleValLeuGluValAlaValLeuSerThrGlu 240  
  
721 GCACAGATCCAAAGACTTAAATTTCCTCTGGCATCAAAGGAGGAGGCCAGTCATCAA 780  
241 GlyGlnIleGlnAspPhyLysPheProLeuGlyIleLysGlyAlaGlySerSerIleGln 260  
  
781 CTGTCGGCAATTACCGTCAAAAGAACACAGGAAATGGCTGCAAGTGGCTTCATC 840  
261 LeuSerAlaAsnThrValLysGlnAsnSerArgAsnGlyLeuValLysLeuValPheIle 280  
  
841 ATTACCGGACCCCTGGACACACTTCCTAGTACAGAAATTGCAACCTTAAACTGGCT 900  
281 IleTyrArgSerLeuGlyGlnPheLeuSerSerGluAsnAlaIleLysLeuGlyAla 300  
  
901 CATTTCATTGCTGTAATAGGACCATTCAGTACAGTAACTCTCACGTCATTCACTTC 960  
301 AspPheIleGlyArgAsnSerThrIleAlaValAsnSerHisValLeuSerValSerIle 320  
  
961 ATAAAGAGTCAGCCGAGTATACCTGACTGATCCTGTGCTTACCTGCCACACATT 1020

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Figure 9

321 AsnLysGluSerSerArgValTyrLeuThrAspProValLeuPheThrLeuProHisIle 340  
1021 GATCCCTGACAAATTTCATAATGCCAAACTGCTCCTTCCTGAACTACTCAGAGACAACTATG 1080  
341 AspProAspAsnTyrPheAsnAlaAsnCysSerPheTrpAsnTyrSerGluArgThrMet 360  
  
1081 ATGGGATATTCGGCTACCCAGGGCTGCAAGCTGGTGAACATAAATAAACTCGAACAAACG 1140  
361 MetGlyTyrTyrSerThrGlnGlyCysLysLeuValAspThrAsnLysSerArgThrThr 380  
  
1141 TGTGGTGGAGCCACCTAACCAATTTCGAAATTCTCATGGCCACAGGGAAATTGCATAT 1200  
381 CysAlaCysSerHisLeuThrAsnPheAlaIleLeuMetAlaHisArgGluIleAlaTyr 400  
  
1201 AAGGATGGCGITCAGTCAATTCTGCACTTCAACCTTCGCTGGAAATTGTCATTTCC 1260  
401 LysAspGlyValHisGluLeuLeuLeuLeuLeuLeuLeuLeuLeuLeuLeuLeu 420  
  
1261 CTGCTTGCTGGCTATCTGCATCTCACCTTCGCTTTCCTGCTGCCTACAGAGTGAC 1320  
421 LeuValCysLeuAlaIleCysIlePheThrPheCysPheArgGlyLeuGlnSerAsp 440  
  
1321 CGAAATACTATTCAGAACCTTGATATCAACCTTTTCATTCGCTGAATTTCCTTA 1380  
441 ArgAsnThrIleHisLysAsnLeuCysIleAsnLeuPheIleAlaGluPheIleLeu 460  
  
1381 ATAGGCCATTGATAAGACAAAATATGCGATTCGCTACCCAAATATTGAGGACTTCACAC 1440  
461 IleGlyIleAspLysThrLysTyrAlaIleAlaCysProIlePheAlaGlyLeuLeuHis 480

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Figure 10

1441	TTTTCTTTGGCAGCTTGCATGCGCTAGAAGGTGTGCAGCTACCTAATG	1500
481	PhePhePheLeuAlaAlaPheAlaTrpMetCysLeuGluGlyValGlnLeuTyrLeuMet	500
1501	TTAGTTGAAGTTTGAAAGTGAATATTCAAGGAAAAATATTACTATGTTGCTGGTTAC	1560
501	LeuValGluValPheGluSerGluTyrSerArgLysLysTyrTyrTyrValAlaGlyTyr	520
1561	TTGTTTCCGCCACAGTGGTTGGAGTTTCAGCTGCTATTGACTATAAGAGCTATGGAACA	1620
521	LeuPheProAlaThrValValGlyValSerAlaAlaIleAspTyrLysSerTyrGlyThr	540
1621	GAAAAAGCTGCTGGCTTCATGTTGATAACTACTTATATGGAGCTTCATTGGACCTGTT	1680
541	GluLysAlaCysTrpLeuHisValAspAsnTyrPheIleTrpSerPheIleGlyProVal	560
1681	ACCTTCATTATTCCTGCTAAATATTATCTTCCTGGTGATCACATTGTGCAAAATGGTGAAG	1740
561	ThrPheIleIleLeuLeuAsnIleIlePheLeuValIleThrLeuCysLysMetValLys	580
1741	CATTCAAACACTTGTAAACAGATTCTAGCAGGPTGGAAAACATTAAAGTCCTGGGTGCTT	1800
581	HisSerAsnThrLeuLysProAspSerSerArgLeuGluAsnIleLysSerTrpValLeu	600
1801	GGCGCTTTCGCTCTCTGTTGTCCTTGGCTCACCTGGCTTTGGTTGCTTTTATT	1860
601	GlyAlaPheAlaLeuLeuCysLeuLeuGlyLeuThrTrpSerPheGlyLeuLeuPheIle	620
1861	AATGAGGAGACTATTGTGATGGCATATCTCTTCACTATATTAAATGCTTCCAGGGAGTG	1920
621	AsnGluGluThrIleValMetAlaTyrLeuPheThrIlePheAsnAlaPheGlnGlyVal	640
1921	TTCATTTCATCTTCACTGTGCTCTCCAAAAGAAAGTACGAAAAGATAATGGCAAGTGC	1980
641	PheIlePheIlePheHisCysAlaLeuGlnLysLysValArgLysGluTyrGlyLysCys	660

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Figure 11

1981	TTCAGACACTCATACTGCTGTGGAGGCCCAACTGAGAGTCCCCAACAGTCAGTGAAAG	2040
661	PheArgHisSerThrCysCysGlyGlyLeuProThrGluSerProHisSerValLys	680
2041	GCGATCAACCAGAACCCAGTCTCGTATTCTCTGGCACACAGAGTCGTATAAGAGA	2100
681	AlaSerThrThrArgThrSerAlaArgTrpSerGlyThrGlnSerArgIleArgArg	700
2101	ATGGTGGAAATGATACTGTGGAGAACATTAGAACTCTCTTTATCTCAGTCAGTCATCAAT	2160
701	MettItpAsnAspThrValArgLysGlnSerGluSerSerPheIleSerGlyAspIleAsn	720
2161	AGGACATTCAAACTTAATCAAGGAATGACTGGCAATTACCTACTAACAAACCCCTCTCTT	2220
721	SerThrSerThrLeuAsnGlnGlyMetThrGlyAsnTyIleLeuThrAsnProLeuLeu	740
2221	CGACCCCACGGCACTAACACCTATAAACATTTGCTCTGGAAACAGTGTTATGTTAAT	2280
741	ArgProHisGlyThrAsnProTyIleAsnProGlyHisSerProGlyIleAsnAsnAlaAspThr	760
2281	GCCCTTCAGCTCCCTGTTAACTTACCTACAGGACATTCACCTGAAACAAATGCCAGGGATACA	2340
761	AlaProSerAlaProValPheAsnSerProGlyHisSerLeuAsnSerIleAsnAsnAlaAspThr	780
2341	ACTGCCATGCGATACTCTACCGCTAAATGGTATTAAACAAACAGCTACTCGCTGCAACAAG	2400
781	SerAlaMetAspThrLeuProLeuAsnGlyAsnPheAsnAsnSerIleSerLeuHisLys	800
2401	GCGGACTATAATGACACGGTGCAGCTGCTGGACTGAGTCGAAATGATACATCTGCT	2460
801	GlyAspTrpAsnAspSerValGlnValValAspCysGlyIleSerLeuAsnAspThrAla	820

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Figure 12

2461 TTTGAGAAAATGATCATTCAGAATTAGTGTACAAACACTAACCTTGAGGTACCCAGCAAGACT 2520  
821 PheGluLysMetIleLeuSerGluLeuValHisAsnAsnLeuArgGlySerSerLysThr 840

2521 CACAACCTCGAGCTCACCGTCAACCTGTCATGGAGCTACCCAGCAACCCAGGGCTGGAGCT 2580  
841 HisAsnLeuGluLeuIleSerProValIleGlyGlySerSerGluAsp 860

2581 GATGCTATTGGCAGATGCCATCTCTATGGACAGGACAACCCAGGGCTGGAGCT 2640  
861 AspAlaIleValAlaAspAlaSerSerLeuMethIleSerAspAsnProGlyIleLeuGluLeu 880

2641 CATCACAGAACTGGGACCACTTATTCCCTAGGGACTCACCCCTCTGTACCAA 2700  
881 HisHisLysGluLeuGluAlaProLeuIleProGlnArgThrHisSerLeuLeuTygGln 900

2701 CCCCGAGAGAAAGTGAAGTCCAGGGAACTGACAGCTATGTCCTCCAACCTGACAGAG 2760  
901 ProGlnLysLysValIleSerGluGlyThrAspSerTyValSerGlnLeutIleAlaGlu 920

2761 CCTGAAAGATCACCTAACAGTCCCCAAACAGAGACTCTCTTATACAGCCATGCCATCTT 2820  
921 AlaGluAspHisLeuGlnSerProAsnArgAspSerIleIlyThrSerMetProAsnLeu 940

2821 AGAGACTCTCCCTATCCGGAGGGAGCCCTGACATGGAAAGAACCTCTCCCTCCACGG 2880  
941 ArgAspSerProTyProGluSerSerProAspMetGluGluAspLeuSerProSerArg 960

2881 AGGAGTGAAGATGAGGACATTTACTATTAAGGATGCCAAATCTTGAGCTGGCCATCAG 2940  
961 ArgSerGluAsnGluAspIleIlyTytytLysSerMetProAsnLeuGlyAlaGlyHisGln 980

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Figure 13

2941	CCTCAGATCTCTTACCAAGATCAGCAGGGCAATAGTGATGGTATAATACTCCCATTAAC	3000
981	LeuGlnMetCysTyrGlnIleSerArgGlyAsnSerAspGlyTyrIleIleProIleAsn	1000
3001	AAAGAAGGGTGTATTCCAGAAGGAGATTTAGAGAAGGACAAATGGAGCTGGTTACAAT	3060
1001	LysGluGlyCysIleProIleGlyAspValArgGluGlyGlnMetGlnLeuValThrSer	1020
3061	CCTTAATCATACAGCTAACGAAATTCCAAGGGCACATGGGAGTAAATAATAAAAGACA	3120
1021	Leu***	1022
3121	CCATTGGCCTGACCCAGCTCCCTCAAACCTCTGCTGAAGAGATGACTCTTGACCTGTGGT	3180
1022		1022
3181	TCTCTGGTGTAAAAAAGATGACTGAAACCTTGGAGTCCTGAAATTTTATAAACATACA	3240
1022		1022
3241	AAAACCTTGATATAACACAGTATACTAAAGTGAATTGAAATTGTTACAAAGAAAAGAGAT	3300
1022		1022
3301	GCCAGCCAGGTATTTAAGATTCCTGCTGCTGTTAGAGAAATTGTGAAACAGCAAAACA	3360
1022		1022
3361	AAACCTTCCAGGCAATTACTGGAGCTGTGAACTAATTGTAAATNTGGCTGGCAC	3420
1022		1022

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Figure 14

3421	CATTITGTAGCCCTGTTTATATAACAGCTTAAATCCTGGGAC	3480	
1022			
3481	AAATTACTGACCTACTATTCCTGAAAGACCTTGAAAGCAGAGATTTCTGCA	3540	
1022			
3541	TCACTTCACTGAAATCTTACATTAGGAANGATTGAAAACATGCTAAC	3600	
1022			
3601	CACTAGCAATCAAGCCACAGCCCTATTCTATATCTTCAACTGACATGAACTAT	3660	
1022			
3661	TCTCTGAAATGGCTAANAAATTATAATTGCTTACATGCTAAATAAATAC	3720	
1022			
3721	AATTGCTGCCAACCTGAAATAATTGTCTTACATGTTAAATTAAGAGTGAAAGAAATAT	3780	
1022			
3781	TGTGCAAAGCTCTTGGTGCACATGTTGAAATGTTTCTTACACTTGTCATGGTA	3840	
1022			
3841	AGTTCTACTCATTTGACTCTTCCACTGTTACAGTTCTGCTTGACAAGTTAG	3900	
1022			
3901	TCTTATACATTAATTCCTTATTGCAAAGAACGTTGTTTATGGGAGAAC	3960	

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Figure 15

1022			
3961	AAACTCTTGAAGCCAGTTATGTCACTGCCTTGACAAAAGTGTGAAATCTAGAAAGAT	4020	
1022			
4021	TGTGTGTACCCCCCTGGTTATCTTGACAGAGGGCAAAGAGGGCACTGGGCACTCTCAC	4080	
1022			
4081	AAACTCTTAGTGAACAAAGGTGCCTATTCTTGACAAAATAAAAACATAAA	4140	
1022			
4141	TATTAATCTCCATATTCCTCTGCCATATTAGTAAATTATTTATGATAAAAGT	4200	
1022			
4201	TCTATGAAATGAAATTCTTCAGGAAATTCTGCCTTTTCATCCCTTGTGAAA	4260	
1022			
4261	CCTCTTAATAATGAGGCCATCACTAATATCCAGTGTAAAGTTAACCGTTGACAGTA	4320	
1022			
4321	AATAAAATGTGAATTTCAGT	4343	
1022			

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Figure 16

1 50  
HK05006 \_\_\_\_\_  
HK05490 \_\_\_\_\_  
HH02631 MARLAAVLWN LCVTAVLVTS ATQGLSRAGL PFGLMRRELA CEGYPIELRC

51 100  
HK05006 \_\_\_\_\_  
HK05490 \_\_\_\_\_  
HH02631 PGSDVIMVEN ANYGRTDDKI CDADPFQMEN VQCYLPDAFK IMSQRCNNRT

101 150  
HK05006 \_\_\_\_\_  
HK05490 \_\_\_\_\_  
HH02631 QCVVVAGSDA FPDPCPGTYK YLEVQYDCVP YKVEQKVFC PGTLQKVLEP

151 200  
HK05006 \_\_\_\_\_  
HK05490 \_\_\_\_\_  
HH02631 TSTHESEHQG GAWCKDPLQA GDRIYVMPWI PYRTDTLTEY ASWEDYVAAR

201 250  
HK05006 \_\_\_\_\_  
HK05490 \_\_\_\_\_  
HH02631 HTTYRLPNR VDGTGFVVYD GAVFYNKERT RNIVKYDLRT RIKSGETVIN

251 300  
HK05006 \_\_\_\_\_  
HK05490 \_\_\_\_\_  
HH02631 TANYHDTSPY RWGGKTDIDL AVDENGLWVI YATEGNNGRL VVSQLNPYTL

301 350  
HK05006 \_\_\_\_\_  
HK05490 \_\_\_\_\_  
HH02631 RFEGTWETGY DKRSASNAFM VCGVLYVLRS VYVDDDSEAA GNRVDYAFNT

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Figure 17

	351		400
HK05006	_____	_____	_____
HK05490	_____	_____	_____
HH02631	NANREEPVSL TFPNPYQFIS SVDYNPRDNQ LYVWNNYFVV RYSLEFGPPD		
	401		450
HK05006	_____	_____	_____
HK05490	_____	_____	_____
HH02631	PSAGPATSSP LSTTTARPT PLTSTASPA A TTPLRRAPLT THPVGAINQL		E
	451		500
HK05006	_____	_____	_____
HK05490	GSKGTKPPPA VSTTKIPPIT NIFPLPERFC EALDSKGIKW PQTQRGMV		E
HH02631	GPDLPATAP VPSTRRPPAP NLHVSPELFC EPREVRRVQW PATQQGMLVE		
	501		550
HK05006	_____	_____	_____
HK05490	RCPKGTRGT ASYLCMISTG TWNPKGPDLS NCTSHVNQL AQKIRSGEN		A
HH02631	RCPKGTRGI ASFQCLPALG LWNPRGPDL S NCTSPWVNQV AQKIKSGEN		
	551		600
HK05006	AEQ TRNHLNAGDI TYSVRAMDQL VGLLDVQLRN LTPGGKDSAA		
HK05490	ASLANELAKH TKGPVFAGDV SSSVRLMEQL VDILDAQLQE LKPSEKDSAG		
HH02631	ANIASELARH TRGSIYAGDV SSSVKLMEQL LDILDAQLQA LRPIERESAG		
	601		650
HK05006	RSLN. .... KAM VETVNNLLQP QALNAWRDLT TSDQLRAATM		
HK05490	RSYNKLQKRE KTCRAYLKAI VDTVDNLLRP EALESWKHMN SSEQAHTATM		
HH02631	KNYNKMHKRE RTCKDYIKAV VETVDNLLRP EALESWKDMN ATEQVHTATM		
	651,		700
HK05006	LLHTVEESAF VLADNLLKTD IVRENTDNK LEVARLSTEG NLEDLKFP. E		
HK05490	LLDTLEEGAF VLADNLLEPT RVSMPTENIV LEVALSTEG QIQDFKFPLG		
HH02631	LLDVLEEGAF LLADNVREPA RFLAAKENVV LEVTVLNT EG QVQE LVFPQE		

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Figure 18

701 750  
HK05006 NMGHGSTIQL SANTLKQNGR NGEIRVAFVL YNNLGPYLST ENASMKLGTE  
HK05490 IKGAGSSIQL SANTVKQNSR NGLAKLVFIY RSLGQFLST ENATIKLGAD  
HH02631 EYPRKNSIQL SAKTIKQNSR NGVVKVVFIL YNNLGLFLST ENATVKLAGE

751 800  
HK05006 A... LSTNHS VIVNSPVITA AINKEFSNKV YLADPVVFTV KHIKQSEENF  
HK05490 F... IGRNST IAVNSHVISV SINKE. SSRV YLDPVLFTL PHI. DPDNYF  
HH02631 AGPGGGPGAS LVVNSQVIAA SINKE. SSRV FLMDPVIFTV AHL. EDKNHF

801 850  
HK05006 NPNCSFWSYS KRTMTGYWST QGCRLTTNK THTTCSCNHL TNFAVLMAHV  
HK05490 NANCSFWNYS ERTMMGYWST QGCKLVDTNK TRTCACSHL TNFAILMAHR  
HH02631 NANCSFWNYS ERSMLGYWST QGCRLVESNK THTTCACSHL TNFAVLMAHR

851 900  
HK05006 EVKHSDAVHD LLLDVITWVG ILLSLVCLLI CIFTFCFFRG LQSDRNTIHK  
HK05490 EIAYKDGVHE LLLTVITWVG IVISLVCLAI CIFTFCFFRG LQSDRNTIHK  
HH02631 EI.YQGRINE LLLSVITWVG IVISLVCLAI CISTFCFLRG LQDRNTIHK

901 950  
HK05006 NLCISLFVAE LLFLIGINRT DQPIACAVFA ALLHFFFIAA FTWMFLEGVQ  
HK05490 NLCINLFAE FIFLIGIDKT KYAIACPIFA GLLHFFFIAA FAWMCLEGVQ  
HH02631 NLCINLFLAE LLFLVGIDKT QYEIACPIFA GLLHYFFFIAA FSWLCLEGVH

951 1000  
HK05006 LYIMLVEVFE SEHSRRKYFY LGYGMPALI VAVSAAVDYL SYGTDKVWL  
HK05490 LYLMLEVFE SEYSRKYYYY VAGYLFPATV VGVSAAIIDYK SYGTEKACWL  
HH02631 LYLLLVEVFE SEYSRTKYYYY LGGYCFPALV VGIAAAIDYR SYGTEKACWL

1001 1050  
HK05006 RLDTYFIWSF IGPATLIIML NVIFLGIALY KMFHHTAILK PESGCLDNIK  
HK05490 HVDNYFIWSF IGPVTFIILL NIIFLVITLC KMVKHSNTLK PDSSRLENIK  
HH02631 RVDNYFIWSF IGPVSFVIVY NLVFLMVTIH KMIRSSSVLK PDSSRLDNIK

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Figure 19

	1051	1100
HK05006	SWVIGAIALL CLLGLTWAFG LMYINESTVI MAYLFTIFNS LQGMFIFIFH	
HK05490	SWVLGAFALL CLLGLTWSFG LLFINEETIV MAYLFTIFNA FQGVFIFIFH	
HH02631	SWALGAIALL FLLGLTWAFG LLFINKESVV MAYLFTTFNA FQGVFIFVFH	
	1101	1150
HK05006	CVLQKKVRKE YGKCLR. THC CSGKSTESSI GSGKTSGSRT PGRYSTGSQS	
HK05490	CALQKKVRKE YGKCFRHSHYC CGGLPTESPH SSVKASTTRT SARYSSGTQS	
HH02631	CALQKKVHKE YSKCLRHSYC CIRSPGGTH GSLKTSAMRS NTRYYTGTQS	
	1151	1200
HK05006	RIRRWNNDTV RKQSESSFIT GDINSSASLN REGLLN. . . . .	
HK05490	RIRRWNNDTV RKQSESSFIS GDINSTSTLN QGMTGNYLLT NPLLRLPHGTN	
HH02631	RIRRWNNDTV RKQTESSFMA GDINSTPTLN RGTMGNHLLT NPVLQPRGGT	
	1201	1250
HK05006	..... NARDTS VMDTLPLNGN	
HK05490	NPYNTILLAET VVCNAPSAPV FNSPGHSLN. . . . NARDTS AMDTLPLNGN	
HH02631	SPYNTLIAES VGFPNPSSPPV FNSPGSYREP KHPLGGREAC GMDTLPLNGN	
	1251	1300
HK05006	HGNYSIASG EYLSN. CVQI IDR GYNHNE. TALEKKILKE LTSNYIPSYL	
HK05490	FNNYSLHKD DY. . NDSVQV VDCGLSLND. TAFEKMI ISE LVHN. . . NL	
HH02631	FNNYSLRSG DFPPGDGGPE PPRGRNLADA AAFEKMI ISE LVHN. . . NL	
	1301	1350
HK05006	NNHERSSEQN RNLMNKLVNN LGSGREDDAI VLDDATSFNH EESLGLELIH	
HK05490	RGSSKTHN. . LEITLPVKPV IGGSSSEDDA IVADASSLMH SDNPGLELHH	
HH02631	RGSSSAAKGP PPPEPPVPPP PGGGGEE. . . . EAGGPGG ADRAEIELLY	
	1351	1400
HK05006	EESDAPLLPP RVYSTENHQD HHYTRRRIPQ DHSESFFPLL TNEHTEDLQS	
HK05490	KELEAPLIPQ RTHSL. . . LYQPQKKVKS EGTDSQL TAEAEIDLQS	
HH02631	KALEEPLLPP RAQSV. . . . LYQSD. . . L DESESCTAED GATSRPLSSP	

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Figure 20

1401

1450

HK05006 PHRDSLTYTSM PTLAGVAATE SVTTSTQTE. . . PPPAKCGD AEDVYYKSM.  
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Figure 21

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Figure 22

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Figure 23

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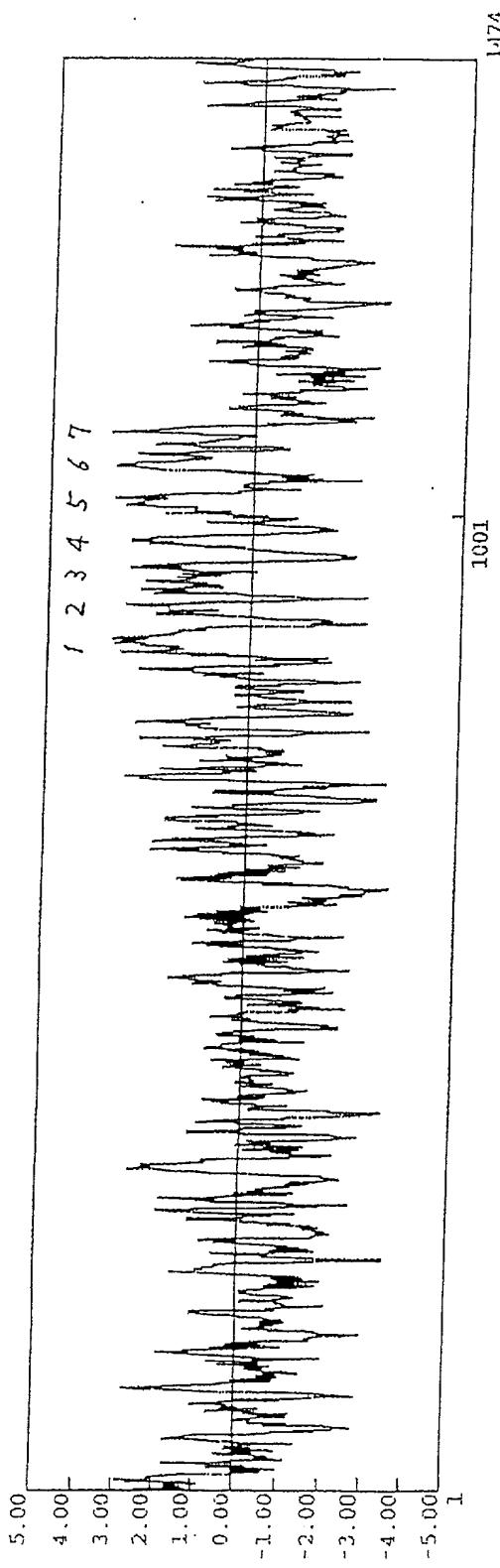
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Figure 25



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J002 Rec'd PCT/PTO 22 JAN 2001  
Docket No.2534USOP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Osamu OHARA et al.  
Serial No. :  
Filed on :  
Group Art Unit :  
Examiner :  
Title : Novel G Protein Coupled Receptor Protein and Its DNA

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

**STATEMENT IN SUPPORT OF FILING  
AND SUBMISSIONS IN ACCORDANCE WITH 37 CFR §§ 1.821-1.825**

In accordance with 37 CFR § 1.821(f), I hereby state that the content of the paper and computer-readable copies of the sequence listing submitted in accordance with 37 CFR §§ 1.821-1.825, respectively, are the same.

Date: January 12, 2001

Respectfully submitted,



Philippe Y. Riesen, Reg. No. 35,657  
Attorney for Applicants  
Customer No. 23,115

09/744226

J602 Rec'd PCT/PTC 22 JAN 2001

## **SEQUENCE LISTING**

<110> Ohara, Osamu  
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<110> Nomura, Nobuo  
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 GGGCCCGATG GGGACGGGCA GATGCAGCTG GTCAACAGTC TC 4425

## Japanese Language Declaration (日本語宣言書)

私は、ここに、以下に記載した外国での特許出願または発明者証の出願、或いは米国以外の少なくとも一国を指定している米国法典第35編第365条(a)によるPCT国際出願について、同第119条(a)-(d)項又は第365条(b)項に基づいて優先権を主張するとともに、優先権を主張する本出願の出願日よりも前の出願日を有する外国での特許出願または発明者証の出願、或いはPCT国際出願については、いかなる出願も、下記の枠内をチェックすることにより示した。

## Prior Foreign Application(s)

外国での先行出願

10-207579	Japan
(Number) (番号)	(Country) (国名)
10-225060	Japan
(Number) (番号)	(Country) (国名)
10-284328	Japan

私は、ここに、下記のいかなる米国法特許出願についても、その米国法典第35編119条(e)項の利益を主張する。

(Application No.) (出願番号)	(Filing Date) (出願日)
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私は、ここに、下記のいかなる米国出願についても、その米国法典第35編第120条に基づく利益を主張し、又米国を指定するいかなるPCT国際出願についても、その同第365条(c)に基づく利益を主張する。また、本出願の各特許請求の範囲の主題が、米国法典第35編第1112条第1段に規定された様で、先行する米国出願又はPCT国際出願に開示されていない場合においては、その先行出願の出願日と本国内出願日またはPCT国際出願との間の期間中に入手出願日と本国内出願日またはPCT国際出願日の間に入手出願が、連邦規則法典第37編規則1.56に定義された特許された情報で、連邦規則法典第37編規則1.56に定義された情報について開示義務があることを承認する。

(Application No.) (出願番号)	(Filing Date) (出願日)
(Application No.) (出願番号)	(Filing Date) (出願日)

私は、ここに表明された私自身の知識に係わる陳述が真実であり、且つ情報と信ずることに基づく陳述が、真実であると信じられることを宣言し、さらに、故意に虚偽の陳述などを行った場合は、米国法典第18編第1001条に基づき、罰金または拘禁、若しくはその四方により処罰され、またそのような故意による虚偽の陳述は、本出願またはそれに対して発行されるいかなる特許も、その有効性に問題が生じることを理解した上で陳述が行われたことを、ここに宣言する。

I hereby claim foreign priority under Title 35, United States Code. Section 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application for which priority is claimed

Priority Not Claimed  
優先権主張なし

23/07/1998	<input type="checkbox"/>
(Day/Month/Year Filed) (出願日／月／年)	
07/08/1998	<input type="checkbox"/>
(Day/Month/Year Filed) (出願日／月／年)	
06/10/1998	

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

(Application No.) (出願番号)	(Filing Date) (出願日)
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I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became effective between the filing date of the prior application and the national or PCT International filing date of application.

(Status: Patented, Pending, Abandoned)  
(現況: 特許許可、係属中、放棄)

(Status: Patented, Pending, Abandoned)  
(現況: 特許許可、係属中、放棄)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

## Declaration and Power of Attorney for Patent Application

### 特許出願宣言書及び委任状

#### Japanese Language Declaration

#### 日本語宣言書

私は、以下に記名された発明者として、ここに下記の通り宣言する：

As a below named inventor, I hereby declare that:

私の住所、郵便の宛先そして国籍は、私の氏名の後に記載された通りである。

My residence, post office address and citizenship are as stated next to my name.

下記の名称の発明について、特許請求範囲に記載され、且つ特許が求められている発明主題に関して、私は、最初、最先且つ唯一の発明者である（唯一の氏名が記載されている場合）か、或いは最初、最先且つ共同発明者である（複数の氏名が記載されている場合）と信じている。

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Novel G Protein Coupled Receptor Protein and  
Its DNA

上記発明の明細書はここに添付されているが、下記の都がチェックされている場合は、この限りでない：

の日に出願され、  
この出願の米国出願番号またはPCT国際出願番号は、  
\_\_\_\_\_ であり、且つ  
\_\_\_\_\_ の日に補正された出願（該当する場合）

the specification of which is attached hereto unless the following box is checked:

was filed on July 22, 1999  
as United States Application Number or  
PCT International Application Number  
PCT/JP99/03909 and was amended on  
(if applicable)

私は、上記の補正書によって補正された、特許請求範囲を含む上記明細書を検討し、且つ内容を理解していることをここに表明する。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

私は、連邦規則法典第37編規則1.56に定義されている、特許性について重要な情報を開示する義務があることを認める。

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56

Burden Hour Statement: This form is estimated to take 0.4 hours to complete. Time will vary depending upon the need of the individual case. Any comments on the amount of time you are required to complete this form should be sent to Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner of Patents and Trademarks, Washington, DC 20231

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### Japanese Language Declaration (日本語宣言書)

委任状： 私は本出願を審査する手続を行い、且つ米国特許商標庁との全ての業務を遂行するために、記名された発明者として、下記の弁護士及び／または弁理士を任命する。（氏名及び登録番号を記載すること）

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith (list name and registration number)  
Philippe Y. RIESEN (Reg. No. 35,657), Mark CHAO (Reg. No. 37,293)

書類送付先

Send Correspondence to:

Customer No. 23,115

直通電話連絡先：（氏名及び電話番号）

Direct Telephone Calls to: (name and telephone number)

Mark CHAO  
Takeda Pharmaceuticals America, Inc.  
Phone:(847)383-3372 Fax:(847)383-3481

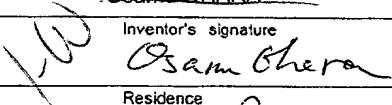
唯一または第一発明者氏名

Full name of sole or first inventor

Osamu OHARA

発明者の署名

日付


November 22, 2000

Date

住所

Residence

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国籍

Citizenship

Japan

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Post Office Address

Kazusa DNA Research Institute

1532-3, Yana, Kisarazu-shi, CHIBA 292-0812 JAPAN

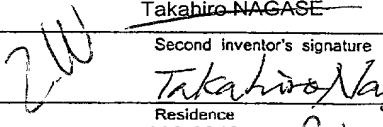
第二共同発明者がいる場合、その氏名

Full name of second joint inventor, if any

Takahiro NAGASE

第二共同発明者の署名

日付


Nov. 24, 2000

Date

住所

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(第三以下の共同発明者についても同様に記載し、署名をすること）

(Supply similar information and signature for third and subsequent joint inventors.)

Attached Sheet to the Declaration

第三共同発明者		Full name of third joint inventor, if any <u>Nobuo NOMURA</u>	
発明者の署名	日付	Third inventor's signature	date
		<i>Nobuo Nomura</i> November 24, 2000	
住所	Residence		
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Kazusa DNA Research Institute, 1532-3, Yana, Kisarazu-shi, CHIBA 292-0812 JAPAN			
第四共同発明者		Full name of fourth joint inventor, if any <u>Shuji HINUMA</u>	
発明者の署名	日付	Fourth inventor's signature	date
		<i>Shuji Hinuma</i> November 17, 2000	
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第五共同発明者		Full name of fifth joint inventor, if any <u>Ryo FUJII</u>	
発明者の署名	日付	Fifth inventor's signature	date
		<i>Ryo Fujii</i> November 30, 2000	
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第六共同発明者		Full name of sixth joint inventor, if any <u>Osamu KITAHARA</u>	
発明者の署名	日付	Sixth inventor's signature	date
		<i>Osamu Kitahara</i> December 9, 2000	
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第七共同発明者		Full name of seventh joint inventor, if any <u>Shinichi MOGI</u>	
発明者の署名	日付	Seventh inventor's signature	date
		<i>Shin-ichi Mogi</i> November 17, 2000	
住所	Residence		
17-16, Mizukino 1-chome, Moriya-machi, Kitasoma-gun IBARAKI 302-0121 JAPAN			
国籍	Citizenship		
Japan			
郵便の宛先	Post Office Address		
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